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Quantitative proteomics provides an insight into germination-related proteins in the obligate biotrophic plant pathogen *Spongospora subterranea*

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Running title: Proteomics analysis of *Spongospora subterranea*

Originality-Significance Statement

The work presented here is the first proteomic analysis of resting spore germination of the obligate biotrophic protozoan plant pathogen *Spongospora subterranea* f.sp. *subterranea*. Infections of potato tubers and roots by *S. subterranea* results in powdery scab and root diseases. Losses due to infections with *S. subterranea* are substantial in most potato growing regions of the world with no fully effective treatments available. The resting spores of *S. subterranea* can remain viable in the soil in their dormant states for decades rendering fields unsuitable for planting. Understanding the molecular basis of resting spore germination might

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lead to the development of new control strategies that either encourage resting spore germination in absence of a host plant leading to soil inoculum depletion, or prevent resting spore germination.

A series of preliminary experiments were undertaken for *S. subterranea* sporosori enrichment and optimisation of protein preparation. Through these analyses, we demonstrated that partial purification of sporosori using Ludox® gradient density centrifugation followed by filter-based digestion of extracted proteins enable efficient proteomics analysis of *S. subterranea*. These findings provide the first insights into the basic protein components of *S. subterranea* spores, a better understanding of the metabolic processes associated with resting spore germination and provide a model for analysis of similar recalcitrant obligate soil-borne organisms.

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Summary

The soil-borne and obligate plant-associated nature of *S. subterranea* has hindered a detailed study of this pathogen and in particular the regulatory pathways driving the germination of *S. subterranea* remain unknown. To better understand the mechanisms that control the transition from dormancy to germination, protein profiles between dormant and germination stimulant-treated resting spores were compared using label-free quantitative proteomics. Among the ~680 proteins identified 20 proteins were found to be differentially expressed during the germination of *S. subterranea* resting spores. Elongation factor Tu, histones (H2A and H15), proteasome and DJ-1_PfpI, involved in transcription and translation, were upregulated during the germination of resting spores. Downregulation of both actin and beta-tubulin proteins occurred in the germinating spores, indicating that the changes in the cell wall cytoskeleton may be necessary for the morphological changes during the germination of the resting spore in *S. subterranea*. Our findings provide new approaches for the study of these and similar recalcitrant micro-organisms, provide the first insights into the basic protein components of *S. subterranea* spores. A better understanding of *S. subterranea* biology and may lead to the development of novel approaches for the management of persistent soil inoculum.

Keywords: *Spongospora subterranea*; resting spores; germination; proteomic

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Introduction

Plasmodiophorids are obligate biotrophic plant pathogens that can infect a wide variety of commercially important crops (Neuhauser *et al*., 2010). Since they produce spores, historically, the plasmodiophorids have been allied with fungi (Waterhouse, 1973). However, the phylogenetic analyses of small subunit ribosomal RNA genes have revealed that these organisms are not true fungi, but rather belong to the protozoan phylum *Cercozoa* (Castlebury and Domier, 1998; Cavalier-Smith and Chao, 2003). *Plasmodiophora brassicae*, the cause of clubroot disease in cruciferous plants, is the best-studied plasmodiophorid (Dixon, 2014). Other plasmodiophorids include *Spongospora subterranea* f.sp. *subterranea*, *Polymxa graminis, Sorodiscus callitrichis* and *Polymyxa betae* (Bass *et al*., 2018). The production of long-lived resting spores is a common feature of the plasmodiophorids. However, resting spore morphology varies between members of the plasmodiophorid genera (Cavalier-Smith and Chao, 2003). While a few of them, such as *Plasmodiophora*, produce individual non-aggregated resting spores, many other members, such as *Spongospora* and *Polymxa*, produce sporosori comprised of aggregations of hundreds of resting spores (Kageyama and Asano, 2009; Tamada and Asher, 2016). Previous studies in *Plasmodiophora brassica* showed that both dormant and non-dormant resting spores exist in the population. While non-dormant spores need only a favourable environment, dormant spores require an external germination stimulant which can be the host root exudate or nutrient solution (Neuhauser *et al*., 2010). Among the plasmodiophorids, *S. subterranea* has the most complex sporosori (Falloon *et al*., 2011).

Germination is a remarkable process that provides a mechanism for breaking the dormancy of resting spores. It plays an essential role in the pathogenesis of soil-borne disease and is also a key target for disease management (Balendres *et al*., 2016). Resting spore germination is triggered by the presence of stimulants such as amino acids or sugars in the environment that

activate specialized receptors within the spore. Crucially, spore germination is associated with loss of resistance properties such that the released or germinating structures are more labile and may more easily perish (Krawczyk *et al*., 2016; Balendres *et al*., 2017). Understanding the molecular basis of resting spore germination is therefore valuable to develop control strategies that either encourage resting spore germination in absence of a host plant leading to soil inoculum depletion (Balendres *et al.*, 2017), or to prevent spore germination altogether eliminating release of infective forms of the pathogen (Setlow, 2014). The transition from dormant to germination phase involves wide-ranging physiological adaptation, such as rapid swelling and changes in surface properties and cell wall composition (Hollomon, 1970). At the same time enhanced rates of metabolic activities such as protein synthesis and trehalose breakdown can also be detected (Cooper *et al*., 2007). For example, a noticeable change in cell wall composition was reported during the germination of *Mucor rouxii* including the formation of a large central vacuole and a new cell wall (Bartnicki-Garcia *et al*., 1968).

Proteomics approaches based on mass spectrometry analysis are useful for studying the protein profiles of plant pathogens. The pre-infection processes during the early stages of infection are essential periods of the lifecycle of the obligate biotrophic pathogen. Until now most proteomics-based analysis of plant-biotrophic pathogen interactions have focused on resistance responses of the plant host (Lee *et al*., 2009; Lan *et al*., 2019) and few studies have investigated the pre-penetrative events that occur before these pathogens colonise host plant roots (Bheri *et al*., 2019; Pham *et al*., 2019). Detailed knowledge of the molecular events occurring during spore germination of *S. subterranea* could reveal new targets for control strategies focused on the pre-infection phase. However, strategies to achieve this are hindered by the lack of methods for laboratory-based culture of these obligate biotrophs. Thus, the application of high-throughput omics approaches to study the biologically significant life

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stages of these organisms has been limited. Standard extraction of *S. subterranea* material relies on excising lesions from powdery scab-infected potato tubers. The proteomic analysis of such samples is technically challenging, as the pathogen proteins are contaminated by proteins from the host plant and other rhizosphere microorganisms. We recently reported an optimized method for sporosori purification and protein extraction for proteomic analysis of *S. subterranea* (Balotf *et al*., 2020). Here, we used this approach, along with data filtering using the known *S. subterranea* proteome to eliminate extraneous peptides, to provide the first proteomic analysis of *S. subterranea* germinating and resting spores. The proteome presented in this study opens new horizons and expands opportunities for studies on spore germination in other obligate biotrophic pathogens.

Results

Validation of the presence of *S. subterranea* in the purified pathogen source

Sporosori from powdery scab lesions collected from infected tubers (Figure 1a) were partially purified using the Ludox® gradient centrifugation protocol. DNA was extracted from two purified samples and PCR was used to detect the *S. subterranea* 18S ribosomal RNA (rRNA) gene with an expected 91 bp product (Figure 1b).

Figure 1

Overview of the *S. subterranea* proteome determined by shotgun proteomics

Label-free quantitative proteomic analyses of germinating and non-germinating resting spores of *S. subterranea* identified a total of 935 proteins. Filtering against the known *S. subterranea* proteome on the UniProt database, based on detection in a minimum of 75% of the samples and at least two unique peptides, reduced the list to 681 proteins. 592 proteins

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were common to both germinating and non-germinating groups, whilst 17 and 72 proteins were detected specifically in germinating and non-germinating spores only, respectively (Figure S1a). This was consistent with the overall slight reduction in the numbers of proteins identified in the germinating spores (618 proteins on average) compared with the non-germinating spores (654 proteins on average) (Figure S1b). A complete list of the identified proteins is presented in supplementary Table S1.

Overall functional annotation of the identified proteins

Gene ontology analysis was used for categorization of the 681 proteins into different groups based on molecular function and biological processes (Figure 2). More than 50% of the proteins could be divided into five major functional categories of molecular function, named organic cyclic compound binding, heterocyclic compound binding, ion binding, small molecule binding and hydrolase activity. Other significantly represented categories include carbohydrate derivative binding, oxidoreductase activity, transporter activity and ATP binding (Figure 2, left). Our results indicated that the majority of identified proteins related to metabolic activities. The three largest groups were organic substance metabolic processes, cellular metabolic processes and nitrogen compound metabolic processes (Figure 2, right), with small molecule metabolic processes, biosynthetic processes, protein metabolic processes, nucleobase-containing compounds, catabolic processes and localisation the next most abundant groups represented. These metabolic polypeptides are very abundant soluble proteins and generally well represented in other plant pathogens such as *Phytophthora capsici* (Pang *et al*., 2017), *Colletotrichum acutatum* (Brown *et al*., 2008) and *Botrytis cinerea* (Fernández-Acero *et al*., 2010).

Figure 2

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Pathway analysis of the identified *S. subterranea* proteins revealed a number of proteins associated with carbohydrate degradation (n = 7), amino acid biosynthesis (n = 5), amino acid degradation (n = 5), cofactor biosynthesis (n = 3) and nucleotide-sugar biosynthesis (n = 3) (Figure 3a), amongst others. Further functional classification of the *S. subterranea* proteins identified groups of enzymes belonging to several categories, with hydrolases (EC3) the largest group of enzymes identified (Figure 3b). Other major represented enzyme classes include oxidoreductases (EC1), transferases (EC2) and isomerases (EC5).

Figure 3

Differentially abundant proteins in *S. subterranea* spores in response to germination stimulants

Statistical comparison of the proteomics data identified changes in abundance of 20 proteins in response to germination stimulants, of which seven were upregulated and 13 were downregulated (Figure 4). To establish how the metabolism of *S. subterranea* differed after the germination of resting spores, we used a KEGG-based analysis to classify the differentially expressed proteins into metabolic pathways and cellular processes. Of the upregulated proteins, the most highly modulated protein was elongation factor Tu (EF-Tu) (Table S2). In addition, two histone subunits (H2A and H1) were also modestly increased, providing evidence for increased transcriptional activity during spore germination. Upregulation of the multifunctional protein DJ-1_PfpI was also observed during the germination of *S. subterranea* resting spores which can be related to its role in the regulation of transcription or hydrolase activity *(Zhao et al*., 2014). In contrast, structural proteins such as actin and tubulin showed a decrease in their expression during the germination of *S. subterranea* spores (Figure 4 and Table S2). Of the downregulated proteins, several were

classified as uncharacterised proteins, which is consistent with the overall paucity of functional annotation of the *S. subterranea* proteome.

Figure 4

In addition to those proteins quantified across all samples, we identified sets of proteins that were regarded as either specific to non-germinating spores (15 proteins) or germinating spores (seven proteins) (Figure S2 and Table S3). The non-germinating spore-specific proteins were mostly involved in catalytic activity and may have roles in the maintenance of dormancy whilst germination-specific proteins were mostly involved in metabolic activity and in the initiation of translation. Enrichment analysis of up and downregulated proteins (the differentially expressed proteins were combined with the germination- and non-germination-specific proteins for this analysis) according to their major biological functions are shown in Figure 5. Three main categories of GO classification including biological processes, cellular component and molecular function were analysed to obtain the functional distribution of the *S. subterranea* spores' proteins. Likewise, the most abundant downregulated proteins were distributed by molecular function. Of the 28 significantly downregulated proteins, carbohydrate derivative binding (GO:0097367) was the largest group, while the largest molecular function group for upregulated proteins was organic cyclic compound binding (GO:0097159). Upregulated proteins contributed more to cellular components than downregulated proteins (Figure 5). The most common biological process categories in both germinating and non-germinating spores were associated with primary metabolic and cellular metabolic processes. The most highly enriched biological process categories in upregulated proteins were the organic substance catabolic processes (GO:1901575), cellular metabolic processes (GO:0044237), catabolic processes (GO:0009056) and primary metabolic

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processes (GO:0044238). The proteins related to transport, microtubules-based process and "response to stimulants" were decreased.

Figure 5

Verification of differential proteins using qPCR

According to our proteomic analysis of *S. subterranea* spores, 20 proteins were found to be altered in abundance between germination treatments. Six DEPs were selected at random for further validation by qPCR at the mRNA level. Although the expression level of histone H1 (A0A0H5RJM2) did not significantly differ between the germinating and non-germinating spores, the transcript levels of the other upregulated proteins, DJ-1_PfpI (A0A0H5R546) and proteasome (A0A0H5R4R4) corroborated the data from the LC-MS/MS experiments (Figure 6a). The mRNA levels of actin (A0A0H5RFH9), SH3 domain-containing protein (A0A0H5R8Z1) and ubiquitinyl hydrolase 1 (A0A0H5R6S0) were all decreased during resting spore germination (Figure 6b). These results were consistent with the protein analysis data confirming downregulation of proteins has occurred during resting spore germination.

Figure 6

Discussion

In this study, the molecular response of *S. subterranea* to germination stimulants was analysed at the proteome level. We have added value to our preliminary experiments through LFQ proteomic analysis of spore germination, enabling relative quantification of nearly 700 *S. subterranea* proteins. The full implication of our results will be realized when functional annotation of the detected proteins is available. Among the identified proteins, 20 proteins

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were significantly differentially expressed (adjusted P-value < 0.05) during the germination of *S. subterranea* (Figure 4 and Table S2). Our qPCR analysis showed that protein expression and transcript were correlated for the candidate genes we randomly sampled (Figure 6). Analyses of the proteomic profile of *S. subterranea* revealed that most of the identified proteins are classified as metabolic proteins, involved in carbohydrate, amino acid, lipid, and protein metabolism (Bindschedler *et al*., 2009). Similar profiles have been reported in *C. acutatum* (El-Akhal *et al*., 2013) and *Aspergillus nidulans* (Oh *et al*., 2010). Inspection of the classes of protein that were upregulated during the germination of resting spores revealed a higher abundance of proteins related to protein synthesis (i.e. Ef-Tu) (Table S2, Figure 4 and 5a). The dependence of conidial germination on protein synthesis has been demonstrated in *Neurospora crassa* (Mirkes, 1974), *Aspergillus nidulans* (Osherov and May, 2000) and *Botrytis cinerea* (González-Rodríguez *et al*., 2015). These studies are consistent with our results since we have found EF-Tu as the most upregulated protein, suggesting that increased protein synthesis is required in the early stages of *S. subterranea* spore germination. Protein synthesis is one of the most energy-consuming processes in both eukaryotic and prokaryotic cells (Browne and Proud, 2002). Proteins that mediate translation, such as the elongation factor Tu which is a universally conserved GTPase, are observed in the proteomes of many organisms (Macek *et al*., 2008; Pereira *et al*., 2015).

Our results showed that both protein synthesis and transcription occurs during the germination of *S. subterranea* and are probably required for its progression (Zhou *et al*., 2019). The expression levels of both histone H2A and histone H1 were increased in response to germination stimulants. It has been demonstrated that sporulation defects occur in strains when the expression level of H2A/ H2B-encoding genes is reduced (Norris and Osley, 1987; Tsui *et al*., 1997). The lower amount of H2A/H2B can also prevent proper regulation of the transcription cascade (Govin and Berger, 2009). The upregulation of proteasome-associated

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proteins (proteasome subunit alpha type) provides further evidence for the importance of transcription during spore germination in *S. subterranea.* The upregulation of this protein has been reported in the germination of *Nosema bombycis* spores (Liu *et al*., 2016) and might indicate a large turnover of proteins associated with the germination of resting spores (Seong *et al*., 2008) Wang *et al*. (2011) showed that proteasome inhibitors can affect the pathogenicity of *Magnaporthe oryzae*, the rice blast fungus, and delayed its spore formation as well.

Another interesting protein that showed a significant change in our study was DJ-1_PfpI domain-containing protein which was upregulated during the germination of *S. subterranea* resting spores (Figure 4, 6a and Table S2). DJ-1 domain-containing proteins, known as "DJ-1" or heat-shock proteins (Hsp proteins), are multifunctional proteins that have been reported to be involved in the regulation of transcription and mitochondrial function and having protease and molecular chaperone activities (Zhao *et al*., 2014). Abdallah *et al*. (2016) showed that YhbO and YajL, members of the PfpI/Hsp31/DJ-1 superfamily, are involved in protection against environmental stresses and like DJ-1 and Hsp31, they repair methylglyoxal-glycated proteins. In *Saccharomyces cerevisiae*, DJ-1 family members regulate mitochondrial homeostasis, cell cycle and apoptosis (Bankapalli *et al*., 2020). In *Escherichia coli*, these proteins are shown to perform multiple functions, including acid resistance and holdase chaperone activity (Subedi *et al*., 2011). However, the underlying molecular mechanism involved in cellular processes by DJ-1 domain-containing proteins is poorly understood. The upregulation of DJ-1_PfpI domain-containing protein during the germination of *S. subterranea* is probably related to its role in the regulation of transcription (Zhao *et al*., 2014). A noteworthy finding is that most of the upregulated proteins were involved in the regulation of transcription and RNA synthesis which raises the possibility that

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the source of the transcripts required for translation during the germination of *S. subterranea* resting spores could be derived from newly synthesized mRNA (Segev *et al*., 2012).

Some major structural proteins have been characterized in this study. We found that both actin and beta-tubulin proteins were significantly downregulated during *S. subterranea* spore germination (Figure 4 and 6b). It has been shown that the distribution of actin patches polarize and depolarize in the germinating spore in *S. cerevisiae* (Kono *et al*., 2005). Similarly, Horio and Oakley (2005) demonstrated the important role of tubulin in the hyphal tip growth of *A. nidulans*. The polarization of actin patches which happen in the early stage of spore germination, maintain their localization to the site of cell surface growth. In line with the above studies, our results indicated that the changes in the actin cytoskeleton are necessary for the morphological changes during the germination of the resting spore in *S. subterranea*. However, not enough is known about *S. subterranea* cell wall composition and further experimental evidence will be required to determine how actin and beta-tubulin are organised within the cell wall.

In spite of the fact that similar to the other biotrophic plant pathogens, *S. subterranea* is reduced in several metabolic pathways (Schwelm *et al*., 2015), our results indicated a significant change in metabolic pathways during the germination of *S. subterranea* resting spores (Figure 5). We also identified several proteins that showed expression in non-germinating spores but were absent in the germinating spores (Figure S2 and Table S3). Argininosuccinate synthase is one of the non-germination-specific proteins identified in the present study. This enzyme participates in metabolic pathways that are linked to the varied uses of the amino acid arginine. The phosphorylation/dephosphorylation of arginine is shown to be related to spore germination in bacteria (Zhou *et al*., 2019). In fact, arginine phosphomodification can mediate the degradation of spore proteins that need to be removed to enable germination (Trentini *et al.*, 2016). Acetyl-coenzyme A synthetase, a key molecule

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that participates in many biochemical reactions in carbohydrate, lipid, amino acid, and protein metabolism, is another non-germination-specific candidate protein. In *Colletotrichum higginsianum*, acetyl-coenzyme A synthetase gene（*ChAcs*) has been shown to be essential for carbon utilization, lipid metabolism and virulence (Gu *et al*., 2019). Before germination, during the dormant phase, the pathogens must utilize nutrient sources present in the spore, such as lipids, trehalose and glycogen (Wilson and Talbot, 2009). Both glycogen and trehalose break down into glucose and then to pyruvate. In mitochondria, pyruvate is converted into acetyl-coenzyme A, which is required for the tricarboxylic acid (TCA) cycle (Salazar *et al*., 2009).

In conclusion, our findings here represent a significant advance in our understanding of the germination of *S. subterranea* resting spores. We have revealed that protein and RNA synthesis occurred during the germination of spores in *S. subterranea*. We have also identified several candidates for resting spore- and germination- specific proteins that are likely to take part in the maintenance of dormancy of resting spores as well as the virulence of *S. subterranea*. The upregulation of the multifunctional protein DJ-1 is one of the major findings of the present experiment. However, how this protein is involved in the subsequent steps of spore germination is another important question that should be addressed in future studies. Not only helping to gain a better understanding of the germination process of *S. subterranea*, the work presented here is also a steppingstone on the way to further study of the biology of other obligate biotrophic pathogens.

Experimental Procedures

Sporosori collection and purification

Spongospora subterranea sporosori were excised from powdery scab infected potato tubers, collected from commercial fields in Devonport, Tasmania, Australia. The infected tubers

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were first washed with running tap water, rinsed in sterile water, and then air-dried for 3 days. All scab lesions were carefully excised using a scalpel and dried at 40 °C for 3 days. The sporosori were further purified using Ludox® (HS-40 colloidal silica, Sigma, NSW, Australia) density gradient centrifugation as previously described (Balotf *et al*., 2020). Briefly, 100 mg of dried *S. subterranea* sporosori were macerated in 3 mL sterile water and filtered through two layers of cheesecloth. Two mL of filtrate was layered onto 9 mL of Ludox®, 2 mL distilled water added on the top and centrifuged at 4200 ×g for 15 min. Three mL of the uppermost band including *S. subterranea* sporosori was diluted with 40 mL of sterile water and centrifuged as before for 8 min. The resultant pellet was used for protein extraction. PCR was performed with primer pairs targeted to the 18S rRNA gene to confirm the presence of *S. subterranea* in purified samples following the method of (Hernandez Maldonado *et al*., 2013).

Resting spore germination and protein extraction

Sporosori was purified from 100 mg of dried lesions suspended in 1 mL of Hoagland's solution (Balendres *et al*., 2018) and incubated at 25 °C in the dark to induce the germination of resting spores. Sporosori samples incubated at 25 °C in the dark in absence of a germination stimulant served as the non-germination treatment. Sub-samples from stimuli-treated sporosori samples were taken daily and observed microscopically (200 - 400 X) for the presence of active zoospores. The sporosori were harvested for protein extraction when zoospores were detected in all four replicates. Purified sporosori from germination and non-germination treatments were separately lysed with 250 µL of SDS lysis buffer (5% SDS and 50 mM ammonium bicarbonate) and 25 µL of protease inhibitor cocktail (cOmplete Mini EDTA-free; Roche Diagnostics, NSW, Australia) using PowerBead tubes, ceramic 2.8 mm (Qiagen, Hilden, Germany). The mixture was homogenised three times for 60 s each using a

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Fast Prep-24 bead beater (Mp Biomedicals, Seven Hills, NSW, Australia) at 4000 ×g at room temperature with 30 s intervals between runs. Lysates were then clarified by centrifugation at 16000 ×g for 10 min. To the collected supernatant, 6 volumes of ice-cold acetone was added, and tubes incubated at -20 °C overnight to precipitate total proteins. Following incubation, the tubes were centrifuged at 16000 ×g for 10 min and pellets were washed three times with chilled acetone. The protein pellets were then resuspended in 50 µL of SDS solubilization buffer containing protease inhibitor. The experiment was performed with four independent biological replicates with the treatment groups.

Trypsin digestion via the S-Trap filters and off-line desalting

Protein samples were quantified using the Pierce™ 660nm protein assay reagent containing ionic detergent compatibility reagent (Thermo Fisher Scientific, MA, USA). 50 µg of total protein was reduced by adding 20 mM dithiothreitol, boiled at 95 °C for 10 min, cooled to room temperature and alkylated with 40 mM iodoacetamide in the dark for 30 min. Samples were then prepared according to the S-Trap microcolumns (Protifi, Farmingdale, N.Y., USA) manufacturer's instructions. In brief, samples were acidified with a final concentration of 1.2% phosphoric acid. To the samples was added six volumes of binding buffer (100 mM ammonium bicarbonate in 90 % aqueous methanol, pH 7.1) and each sample loaded onto an S-Trap micro spin column after gentle mixing. S-Trap tubes were spun at 4000 rpm for 1 min and washed three times with 150 µL of S-Trap buffer. Samples were then digested with trypsin (2 µg of sequencing-grade trypsin in 20 µL of 50 mM ammonium bicarbonate) for 1 h at 47 °C. The digested peptides were eluted using 50 mM ammonium bicarbonate and then 0.2% formic acid. Samples were further cleaned by off-line desalting using Millipore ZipTips (Merck, Darmstadt, Germany) according to the manufacturer's instructions and dried

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peptides were reconstituted in HPLC loading buffer (2% acetonitrile and 0.05% TFA in water).

LC-MS/MS analysis

Peptides (1 µg of each digest) were separated and analysed using an Ultimate 3000 nano RSLC system (Thermo Fisher Scientific, MA, USA). Tryptic peptides were first concentrated on a PepMap 100 C18 trapping column (particle size, 3 µm; length, 20 mm; diameter, 75 µm) for 5 min. Peptides were then separated using a PepMap 100 C18 analytical column (particle size, 2 µm; length, 250 mm; diameter, 75 µm) at a flow rate of 300 nL/min and held at 45 °C. A 90-minute gradient from 98% mobile phase A (0.1% formic acid in water) to 50% mobile phase B (0.08% formic acid in 80% acetonitrile and 20% water) comprised the following steps: 2-10% B over 12 min, 10-25% B over 48 min, 25-45% B over 10 min, holding at 95% B for 5 min then re-equilibration in 2% B for 15 min. The nanoHPLC system was coupled to a Q-Exactive HF mass spectrometer equipped with nanospray Flex ion source (Thermo Fisher Scientific, MA, USA) and controlled using Xcalibur 4.1 software. Spray voltage was set to 2.0 kV, S-lens RF level to 50, and heated capillary set at 250 °C. MS scans were acquired from 370-1500 m/z at 60,000 resolution, with an AGC target of 3×10^6 and a maximum fill time of 100 ms. Fragment ion scans were acquired at 15,000 resolution (scan range 200-2000 m/z), with an AGC target of 2×10^5 and a maximum fill time of 28 ms. An isolation width of 1.4 m/z was used, and normalized collision energy for HCD set to 27. MS/MS spectra were acquired in data-dependent mode using a Top15 method with 30-second dynamic exclusion of fragmented peptides. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol *et al.*, 2019) partner repository with the dataset identifier PXD022089 (Username: reviewer_pxd022089@ebi.ac.uk; Password: fT5h435B).

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Protein identification and analysis of abundance data

The MS/MS raw data were imported into MaxQuant software (v. 1.6.0.16, www.maxquant.org) for label-free quantitative comparison between germinating and resting spores. Searches were conducted using the Andromeda search engine with default search settings against the *S. subterranea* database downloaded from UniProt (www.uniprot.org/proteomes/,containing 11,129 proteins). Searches were restricted to a peptide false discovery rate (FDR) of 1% for both peptide-spectrum matches and protein identification. The match-between-runs function was enabled. Statistical analysis of LFQ data was performed by LFQ Analyst (Shah *et al*., 2019) and the Perseus software (v. 1.5.0.15, www.perseus-framework.org). Protein groups identified either as potential contaminants, by reverse database matching or identified "only by site" were removed. The default data processing workflow used in LFQ-Analyst only retains proteins that have 75% or more valid values and identified based on at least two unique peptides. Missing values were imputed using the normal distribution of protein abundances left-shifted by 1.8 standard deviation with a width of 0.3. A Benjamini–Hochberg test (Benjamini and Hochberg, 1995) was used to identify the differentially abundant proteins between germinating spores and control samples. Proteins with a fold-change of 1.3 times or more and the FDR of 0.05 or less were defined as quantitatively significant. The proteinGroups.txt output file is presented in Supplemental Tables S4.

Annotation and Gene Ontology (GO) Enrichment Analysis

The protein sequences of each accession number in *S. subterranea* proteome were obtained using the Basic Local Alignment Search Tool (BLAST). The functional categorization was obtained from the GO terms of each protein according to the UniProt database (www.uniprot.org) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) entries

(www.genome.jp/kegg/). Enzyme Commission (EC) was retrieved from the Expasy database (https://enzyme.expasy.org/). Heatmap was drawn using Perseus software and volcano plot was obtained from LFQ Analyst platform (https://bioinformatics.erc.monash.edu/apps/LFQ-Analyst/).

RNA extraction and real-time PCR analysis

To validate the proteomics data, a quantitative real-time PCR (qRT-PCR) analysis was used to measure the mRNA transcript level for the six randomly selected DEPs. For each sample, 100 mg of dried *S. subterranea* sporosori were purified using Ludox® density gradient centrifugation. Half the samples were suspended in 1 mL of Hoagland's solution to stimulate the germination of the spores. Spores were collected 4 days after treatment when motile zoospores were observed in the germinating samples. Two hundred μL of an overnight culture of the marine bacterium *Pseudoalteromonas prydzensis* was added to each sample and used as internal control to confirm RNA quality and to normalise qPCR data. Total RNA from the samples was isolated using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The amount of RNA was determined using a Qubit™ RNA BR Assay Kit (Invitrogen, Waltham, MA). Total RNA was then treated with the DNase I (Qiagen, Hilden, Germany) to remove any remaining DNA from the samples before cDNA synthesis. Primers were designed using the Primer3 (Version 4; www.bioinfo.ut.ee/primer3-0.4.0/) and NCBI Primer-Blast (www.ncbi.nlm.nih. gov /tools/primer-blast). Quantitative real-time PCR experiments were carried out in 20 μL volume in a Qiagen RotorGeneQ (Qiagen, Hilden, Germany) using iTaq Universal SYBR Green Supermix (Bio-Rad, NSW, Australia). Data were normalized using the internal control and the fold change of each gene was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The quantitative gene

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expression analysis were performed with three biological replicates and three technical replicates. The primer sequences were listed in Table S5.

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Figure legends

Figure 1. (a) Powdery scab lesions on potato tubers and (b) agarose gel electrophoresis of the PCR product of 18S rRNA gene for DNA extracted from purified powdery scab materials. Lane M: MassRuler DNA ladder; lane NTC: non-templet control; lane 1 & 2: showing amplified *S. subterranea* 18S rRNA gene in purified samples.

Figure 2. Distribution of identified proteins from the proteome of *S. subterranea* into gene ontology (GO) categories, according to their involvement in biological processes (right) and their molecular function (left).

Figure 3. (a) Pathways analysis and (b) enzyme classes of *S. subterranea* identified proteins.

Figure 4. Comparison of differently abundant proteins in germinating and resting spores of *S. subterranea*. Proteins with FDR < 0.05 are indicated by dark data points and labeled with their respective accession numbers, details of which are provided in Table S2. N-GS: Non-germinating Spores; GS: Germinating Spores.

Figure 5. GO enrichment analysis of (a) the upregulated and (b) downregulated proteins during the germination of *S. subterranea* spores. MF: molecular function; CC: cellular components; BP: biological process.

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Figure 6. Transcript levels of genes encoding (a) upregulated and (b) downregulated proteins. Error bars represent the standard deviation based on three independent biological and three technical replicates. Asterisk (*): significant mRNA difference between germinating and resting spores ($p < 0.05$); 'ns': no significant difference ($p \geq 0.05$); N-GS: Non-germinating Spores; GS: Germinating Spores.

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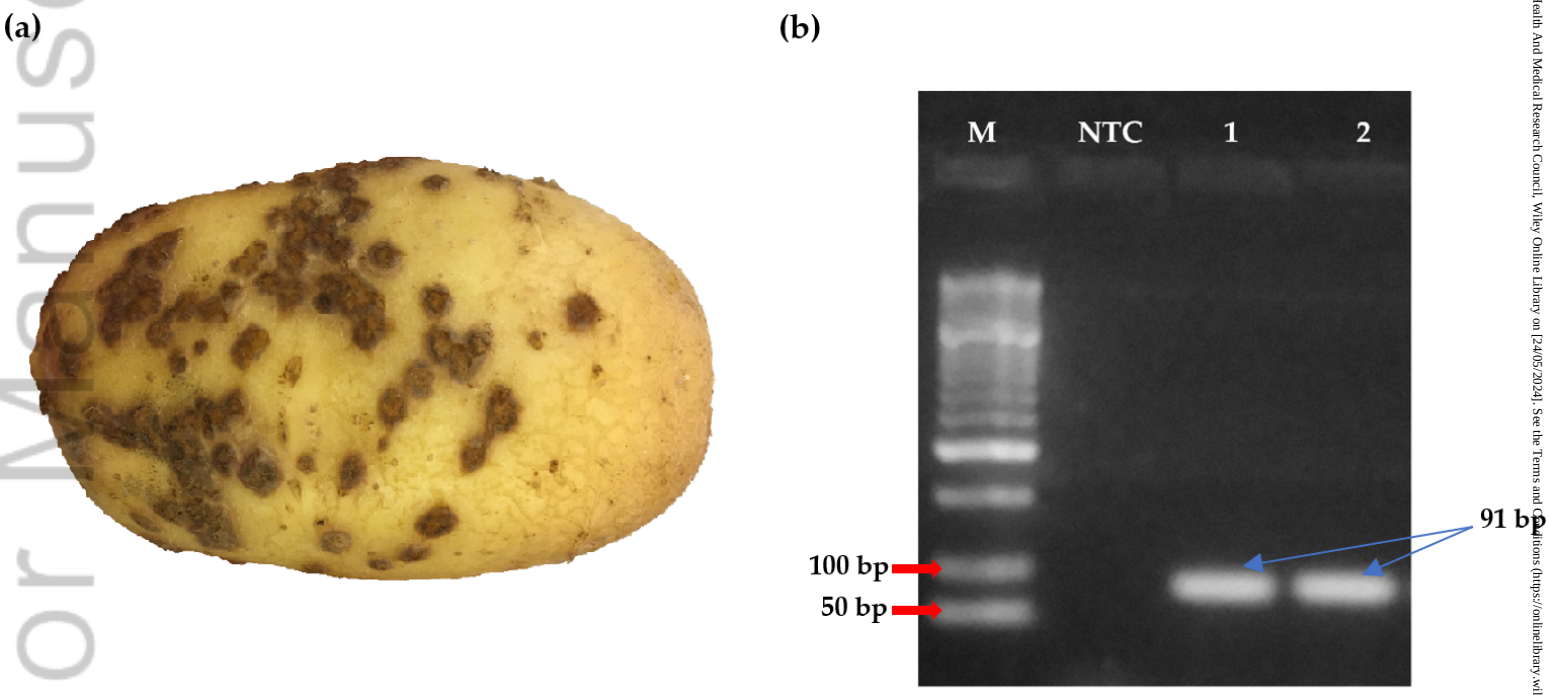

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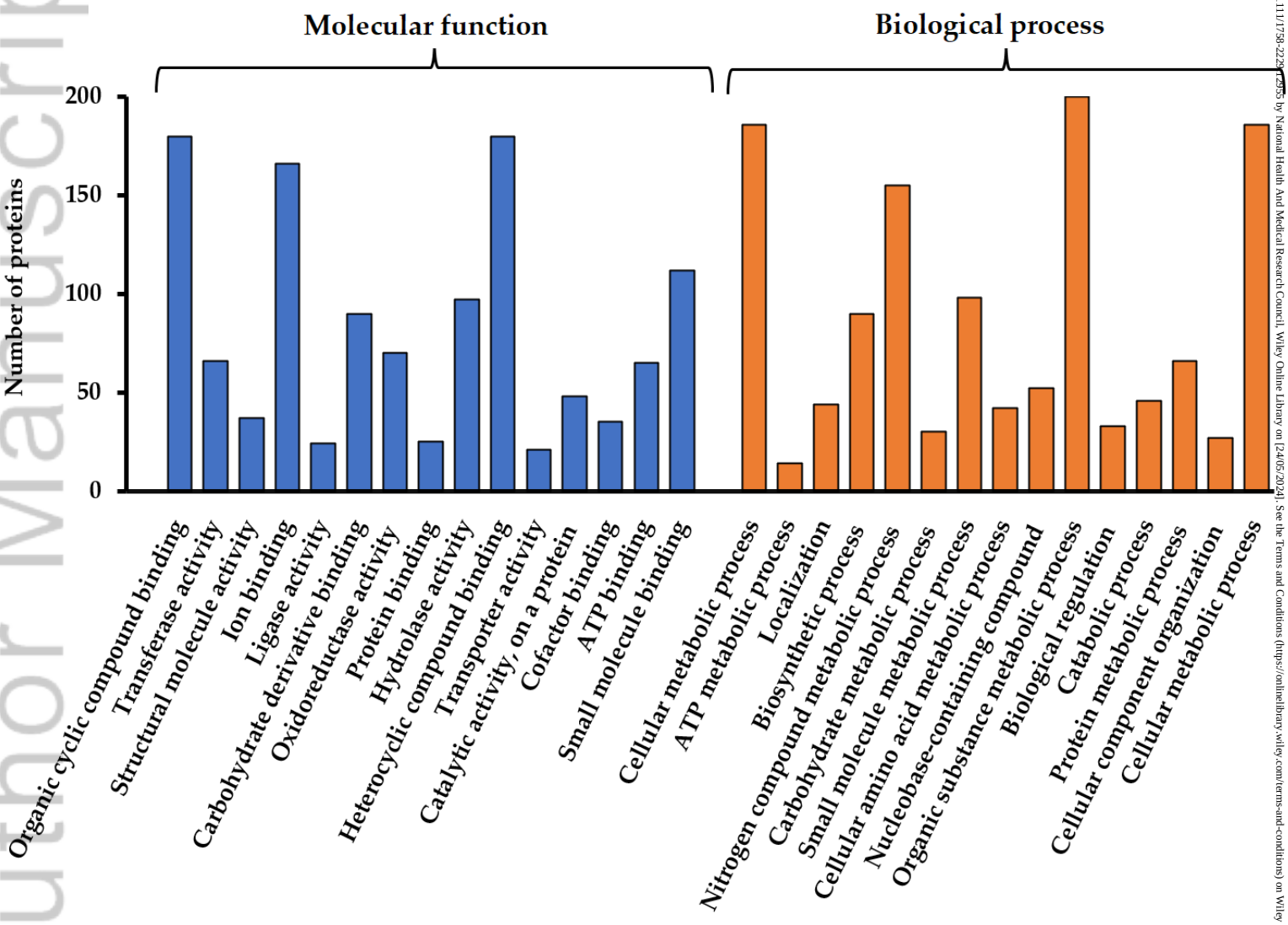

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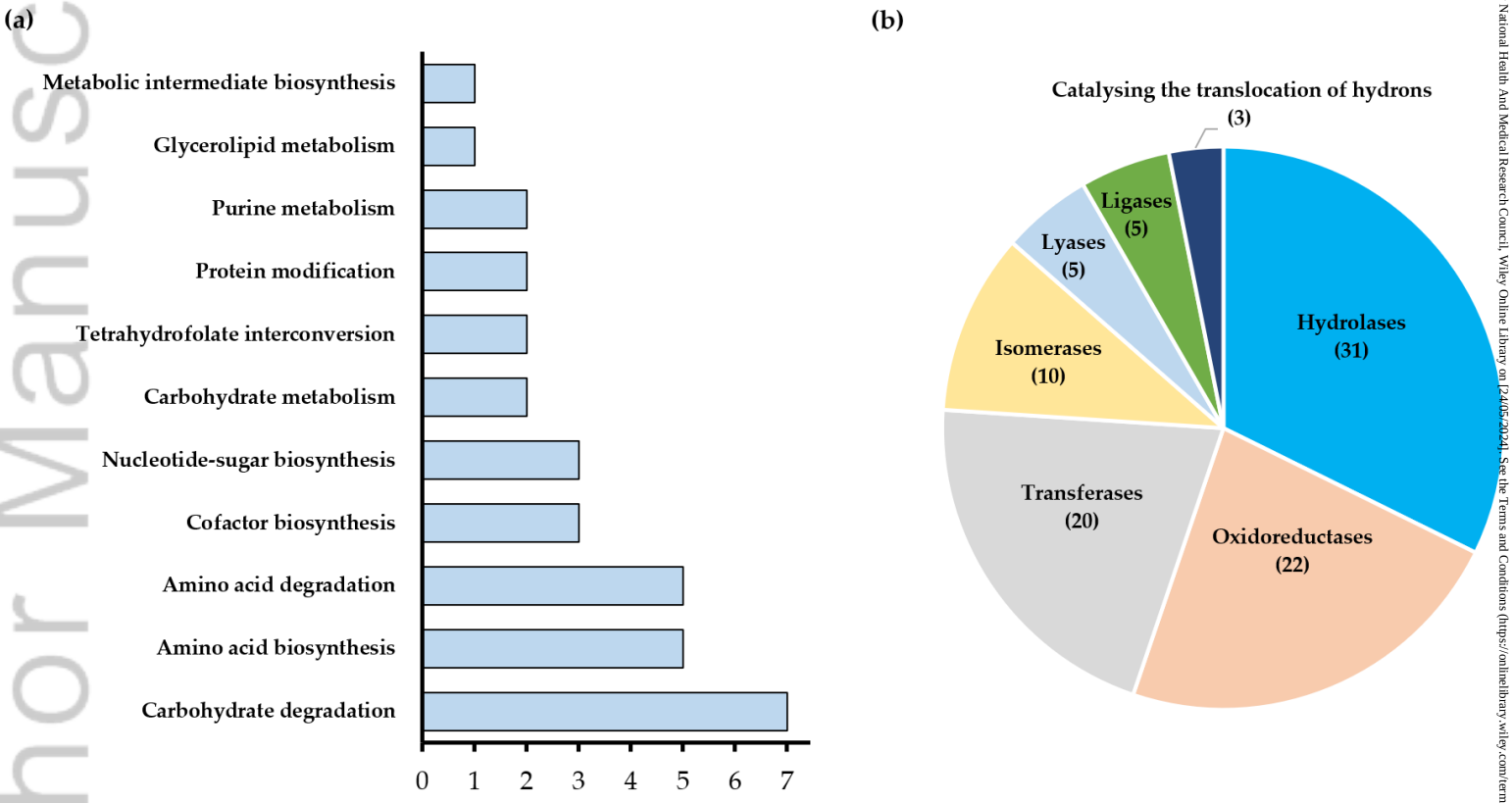

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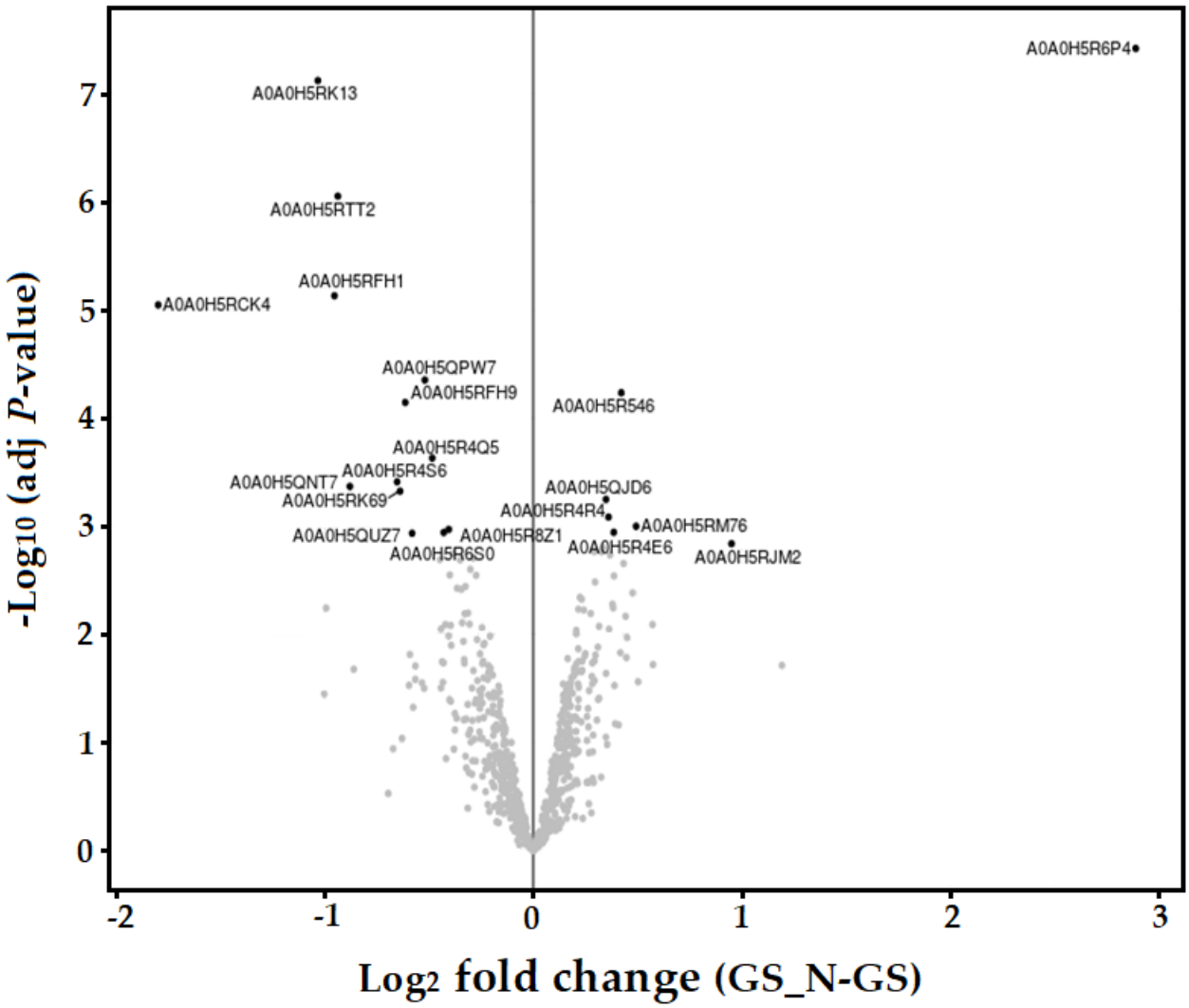

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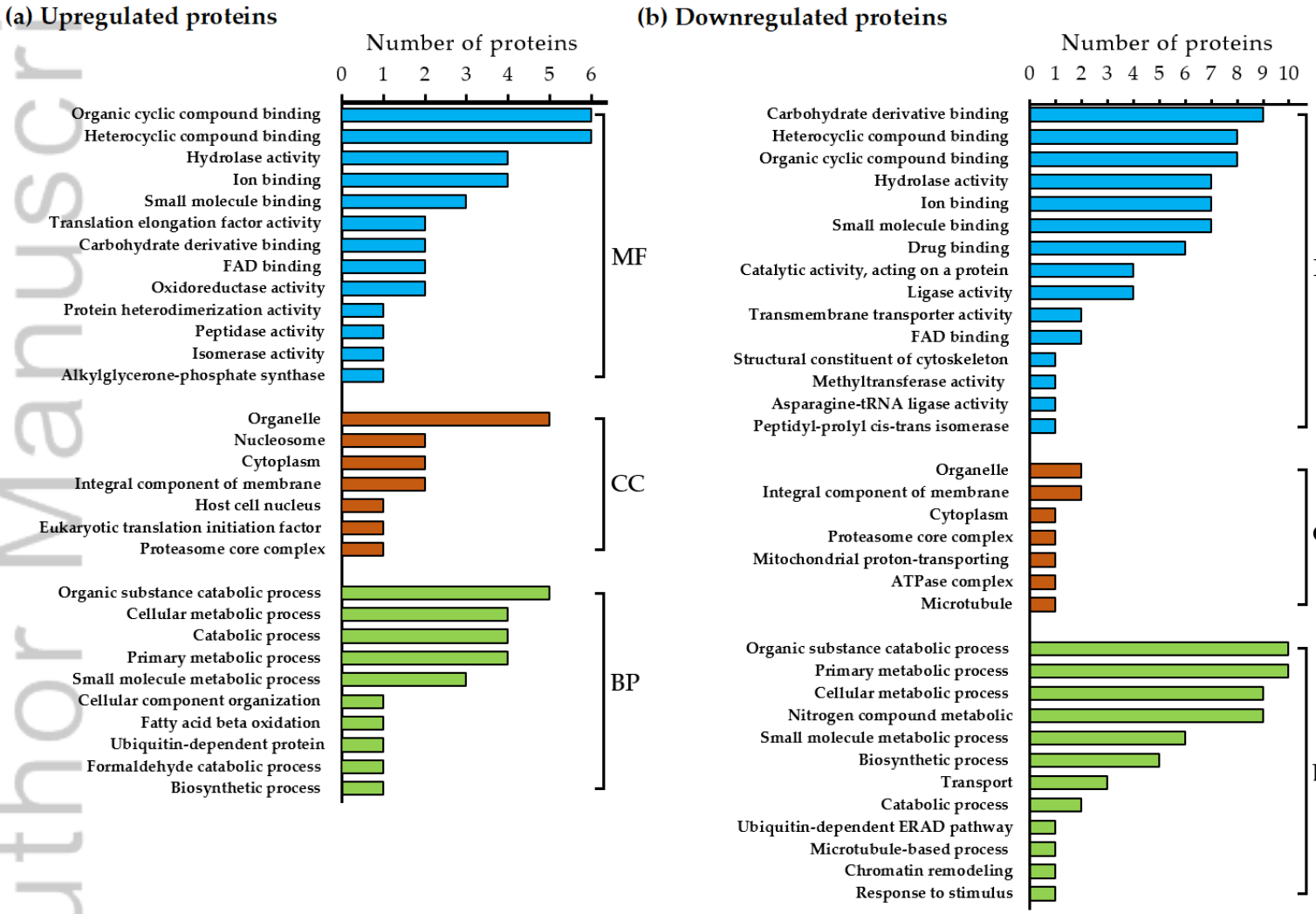

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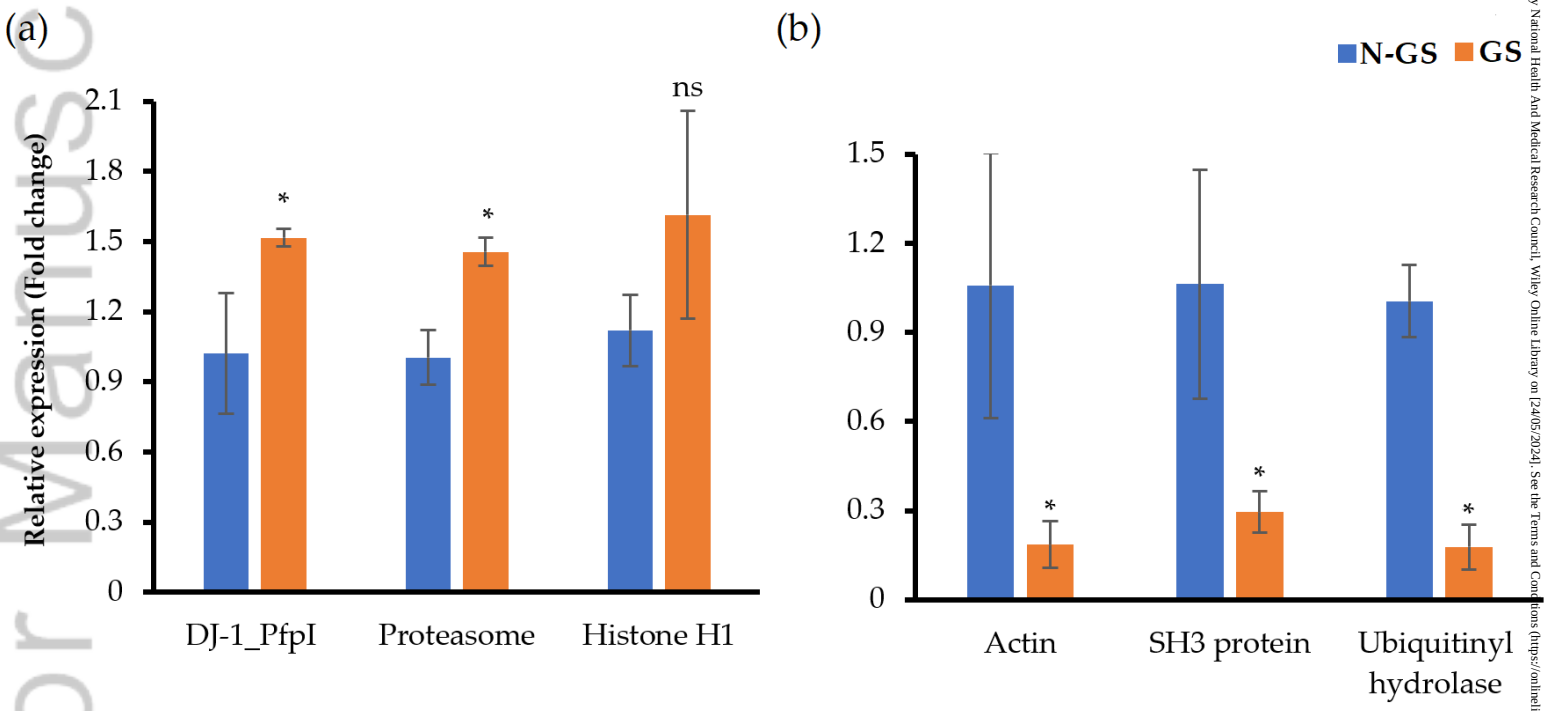

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Quantitative proteomics provides an insight into germination-related proteins in the obligate biotrophic plant pathogen *Spongospora subterranea*

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Running title: Proteomics analysis of *Spongospora subterranea*

Originality-Significance Statement

The work presented here is the first proteomic analysis of resting spore germination of the obligate biotrophic protozoan plant pathogen *Spongospora subterranea* f.sp. *subterranea*. Infections of potato tubers and roots by *S. subterranea* results in powdery scab and root diseases. Losses due to infections with *S. subterranea* are substantial in most potato growing regions of the world with no fully effective treatments available. The resting spores of *S. subterranea* can remain viable in the soil in their dormant states for decades rendering fields unsuitable for planting. Understanding the molecular basis of resting spore germination might

lead to the development of new control strategies that either encourage resting spore germination in absence of a host plant leading to soil inoculum depletion, or prevent resting spore germination.

A series of preliminary experiments were undertaken for *S. subterranea* sporosori enrichment and optimisation of protein preparation. Through these analyses, we demonstrated that partial purification of sporosori using Ludox® gradient density centrifugation followed by filter-based digestion of extracted proteins enable efficient proteomics analysis of *S. subterranea*. These findings provide the first insights into the basic protein components of *S. subterranea* spores, a better understanding of the metabolic processes associated with resting spore germination and provide a model for analysis of similar recalcitrant obligate soil-borne organisms.

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Summary

The soil-borne and obligate plant-associated nature of *S. subterranea* has hindered a detailed study of this pathogen and in particular the regulatory pathways driving the germination of *S. subterranea* remain unknown. To better understand the mechanisms that control the transition from dormancy to germination, protein profiles between dormant and germination stimulant-treated resting spores were compared using label-free quantitative proteomics. Among the ~680 proteins identified 20 proteins were found to be differentially expressed during the germination of *S. subterranea* resting spores. Elongation factor Tu, histones (H2A and H15), proteasome and DJ-1_PfpI, involved in transcription and translation, were upregulated during the germination of resting spores. Downregulation of both actin and beta-tubulin proteins occurred in the germinating spores, indicating that the changes in the cell wall cytoskeleton may be necessary for the morphological changes during the germination of the resting spore in *S. subterranea*. Our findings provide new approaches for the study of these and similar recalcitrant micro-organisms, provide the first insights into the basic protein components of *S. subterranea* spores. A better understanding of *S. subterranea* biology and may lead to the development of novel approaches for the management of persistent soil inoculum.

Keywords: *Spongospora subterranea*; resting spores; germination; proteomic

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Introduction

Plasmodiophorids are obligate biotrophic plant pathogens that can infect a wide variety of commercially important crops (Neuhauser *et al*., 2010). Since they produce spores, historically, the plasmodiophorids have been allied with fungi (Waterhouse, 1973). However, the phylogenetic analyses of small subunit ribosomal RNA genes have revealed that these organisms are not true fungi, but rather belong to the protozoan phylum *Cercozoa* (Castlebury and Domier, 1998; Cavalier-Smith and Chao, 2003). *Plasmodiophora brassicae*, the cause of clubroot disease in cruciferous plants, is the best-studied plasmodiophorid (Dixon, 2014). Other plasmodiophorids include *Spongospora subterranea* f.sp. *subterranea*, *Polymxa graminis, Sorodiscus callitrichis* and *Polymyxa betae* (Bass *et al*., 2018). The production of long-lived resting spores is a common feature of the plasmodiophorids. However, resting spore morphology varies between members of the plasmodiophorid genera (Cavalier-Smith and Chao, 2003). While a few of them, such as *Plasmodiophora*, produce individual non-aggregated resting spores, many other members, such as *Spongospora* and *Polymxa*, produce sporosori comprised of aggregations of hundreds of resting spores (Kageyama and Asano, 2009; Tamada and Asher, 2016). Previous studies in *Plasmodiophora brassica* showed that both dormant and non-dormant resting spores exist in the population. While non-dormant spores need only a favourable environment, dormant spores require an external germination stimulant which can be the host root exudate or nutrient solution (Neuhauser *et al*., 2010). Among the plasmodiophorids, *S. subterranea* has the most complex sporosori (Falloon *et al*., 2011).

Germination is a remarkable process that provides a mechanism for breaking the dormancy of resting spores. It plays an essential role in the pathogenesis of soil-borne disease and is also a key target for disease management (Balendres *et al*., 2016). Resting spore germination is triggered by the presence of stimulants such as amino acids or sugars in the environment that

activate specialized receptors within the spore. Crucially, spore germination is associated with loss of resistance properties such that the released or germinating structures are more labile and may more easily perish (Krawczyk *et al*., 2016; Balendres *et al*., 2017). Understanding the molecular basis of resting spore germination is therefore valuable to develop control strategies that either encourage resting spore germination in absence of a host plant leading to soil inoculum depletion (Balendres *et al.*, 2017), or to prevent spore germination altogether eliminating release of infective forms of the pathogen (Setlow, 2014). The transition from dormant to germination phase involves wide-ranging physiological adaptation, such as rapid swelling and changes in surface properties and cell wall composition (Hollomon, 1970). At the same time enhanced rates of metabolic activities such as protein synthesis and trehalose breakdown can also be detected (Cooper *et al*., 2007). For example, a noticeable change in cell wall composition was reported during the germination of *Mucor rouxii* including the formation of a large central vacuole and a new cell wall (Bartnicki-Garcia *et al*., 1968).

Proteomics approaches based on mass spectrometry analysis are useful for studying the protein profiles of plant pathogens. The pre-infection processes during the early stages of infection are essential periods of the lifecycle of the obligate biotrophic pathogen. Until now most proteomics-based analysis of plant-biotrophic pathogen interactions have focused on resistance responses of the plant host (Lee *et al*., 2009; Lan *et al*., 2019) and few studies have investigated the pre-penetrative events that occur before these pathogens colonise host plant roots (Bheri *et al*., 2019; Pham *et al*., 2019). Detailed knowledge of the molecular events occurring during spore germination of *S. subterranea* could reveal new targets for control strategies focused on the pre-infection phase. However, strategies to achieve this are hindered by the lack of methods for laboratory-based culture of these obligate biotrophs. Thus, the application of high-throughput omics approaches to study the biologically significant life

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stages of these organisms has been limited. Standard extraction of *S. subterranea* material relies on excising lesions from powdery scab-infected potato tubers. The proteomic analysis of such samples is technically challenging, as the pathogen proteins are contaminated by proteins from the host plant and other rhizosphere microorganisms. We recently reported an optimized method for sporosori purification and protein extraction for proteomic analysis of *S. subterranea* (Balotf *et al*., 2020). Here, we used this approach, along with data filtering using the known *S. subterranea* proteome to eliminate extraneous peptides, to provide the first proteomic analysis of *S. subterranea* germinating and resting spores. The proteome presented in this study opens new horizons and expands opportunities for studies on spore germination in other obligate biotrophic pathogens.

Results

Validation of the presence of *S. subterranea* in the purified pathogen source

Sporosori from powdery scab lesions collected from infected tubers (Figure 1a) were partially purified using the Ludox® gradient centrifugation protocol. DNA was extracted from two purified samples and PCR was used to detect the *S. subterranea* 18S ribosomal RNA (rRNA) gene with an expected 91 bp product (Figure 1b).

Figure 1

Overview of the *S. subterranea* proteome determined by shotgun proteomics

Label-free quantitative proteomic analyses of germinating and non-germinating resting spores of *S. subterranea* identified a total of 935 proteins. Filtering against the known *S. subterranea* proteome on the UniProt database, based on detection in a minimum of 75% of the samples and at least two unique peptides, reduced the list to 681 proteins. 592 proteins

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were common to both germinating and non-germinating groups, whilst 17 and 72 proteins were detected specifically in germinating and non-germinating spores only, respectively (Figure S1a). This was consistent with the overall slight reduction in the numbers of proteins identified in the germinating spores (618 proteins on average) compared with the non-germinating spores (654 proteins on average) (Figure S1b). A complete list of the identified proteins is presented in supplementary Table S1.

Overall functional annotation of the identified proteins

Gene ontology analysis was used for categorization of the 681 proteins into different groups based on molecular function and biological processes (Figure 2). More than 50% of the proteins could be divided into five major functional categories of molecular function, named organic cyclic compound binding, heterocyclic compound binding, ion binding, small molecule binding and hydrolase activity. Other significantly represented categories include carbohydrate derivative binding, oxidoreductase activity, transporter activity and ATP binding (Figure 2, left). Our results indicated that the majority of identified proteins related to metabolic activities. The three largest groups were organic substance metabolic processes, cellular metabolic processes and nitrogen compound metabolic processes (Figure 2, right), with small molecule metabolic processes, biosynthetic processes, protein metabolic processes, nucleobase-containing compounds, catabolic processes and localisation the next most abundant groups represented. These metabolic polypeptides are very abundant soluble proteins and generally well represented in other plant pathogens such as *Phytophthora capsici* (Pang *et al*., 2017), *Colletotrichum acutatum* (Brown *et al*., 2008) and *Botrytis cinerea* (Fernández-Acero *et al*., 2010).

Figure 2

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Pathway analysis of the identified *S. subterranea* proteins revealed a number of proteins associated with carbohydrate degradation (n = 7), amino acid biosynthesis (n = 5), amino acid degradation (n = 5), cofactor biosynthesis (n = 3) and nucleotide-sugar biosynthesis (n = 3) (Figure 3a), amongst others. Further functional classification of the *S. subterranea* proteins identified groups of enzymes belonging to several categories, with hydrolases (EC3) the largest group of enzymes identified (Figure 3b). Other major represented enzyme classes include oxidoreductases (EC1), transferases (EC2) and isomerases (EC5).

Figure 3

Differentially abundant proteins in *S. subterranea* spores in response to germination stimulants

Statistical comparison of the proteomics data identified changes in abundance of 20 proteins in response to germination stimulants, of which seven were upregulated and 13 were downregulated (Figure 4). To establish how the metabolism of *S. subterranea* differed after the germination of resting spores, we used a KEGG-based analysis to classify the differentially expressed proteins into metabolic pathways and cellular processes. Of the upregulated proteins, the most highly modulated protein was elongation factor Tu (EF-Tu) (Table S2). In addition, two histone subunits (H2A and H1) were also modestly increased, providing evidence for increased transcriptional activity during spore germination. Upregulation of the multifunctional protein DJ-1_PfpI was also observed during the germination of *S. subterranea* resting spores which can be related to its role in the regulation of transcription or hydrolase activity *(Zhao et al*., 2014). In contrast, structural proteins such as actin and tubulin showed a decrease in their expression during the germination of *S. subterranea* spores (Figure 4 and Table S2). Of the downregulated proteins, several were

classified as uncharacterised proteins, which is consistent with the overall paucity of functional annotation of the *S. subterranea* proteome.

Figure 4

In addition to those proteins quantified across all samples, we identified sets of proteins that were regarded as either specific to non-germinating spores (15 proteins) or germinating spores (seven proteins) (Figure S2 and Table S3). The non-germinating spore-specific proteins were mostly involved in catalytic activity and may have roles in the maintenance of dormancy whilst germination-specific proteins were mostly involved in metabolic activity and in the initiation of translation. Enrichment analysis of up and downregulated proteins (the differentially expressed proteins were combined with the germination- and non-germination-specific proteins for this analysis) according to their major biological functions are shown in Figure 5. Three main categories of GO classification including biological processes, cellular component and molecular function were analysed to obtain the functional distribution of the *S. subterranea* spores' proteins. Likewise, the most abundant downregulated proteins were distributed by molecular function. Of the 28 significantly downregulated proteins, carbohydrate derivative binding (GO:0097367) was the largest group, while the largest molecular function group for upregulated proteins was organic cyclic compound binding (GO:0097159). Upregulated proteins contributed more to cellular components than downregulated proteins (Figure 5). The most common biological process categories in both germinating and non-germinating spores were associated with primary metabolic and cellular metabolic processes. The most highly enriched biological process categories in upregulated proteins were the organic substance catabolic processes (GO:1901575), cellular metabolic processes (GO:0044237), catabolic processes (GO:0009056) and primary metabolic

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processes (GO:0044238). The proteins related to transport, microtubules-based process and "response to stimulants" were decreased.

Figure 5

Verification of differential proteins using qPCR

According to our proteomic analysis of *S. subterranea* spores, 20 proteins were found to be altered in abundance between germination treatments. Six DEPs were selected at random for further validation by qPCR at the mRNA level. Although the expression level of histone H1 (A0A0H5RJM2) did not significantly differ between the germinating and non-germinating spores, the transcript levels of the other upregulated proteins, DJ-1_PfpI (A0A0H5R546) and proteasome (A0A0H5R4R4) corroborated the data from the LC-MS/MS experiments (Figure 6a). The mRNA levels of actin (A0A0H5RFH9), SH3 domain-containing protein (A0A0H5R8Z1) and ubiquitinyl hydrolase 1 (A0A0H5R6S0) were all decreased during resting spore germination (Figure 6b). These results were consistent with the protein analysis data confirming downregulation of proteins has occurred during resting spore germination.

Figure 6

Discussion

In this study, the molecular response of *S. subterranea* to germination stimulants was analysed at the proteome level. We have added value to our preliminary experiments through LFQ proteomic analysis of spore germination, enabling relative quantification of nearly 700 *S. subterranea* proteins. The full implication of our results will be realized when functional annotation of the detected proteins is available. Among the identified proteins, 20 proteins

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were significantly differentially expressed (adjusted P-value < 0.05) during the germination of *S. subterranea* (Figure 4 and Table S2). Our qPCR analysis showed that protein expression and transcript were correlated for the candidate genes we randomly sampled (Figure 6). Analyses of the proteomic profile of *S. subterranea* revealed that most of the identified proteins are classified as metabolic proteins, involved in carbohydrate, amino acid, lipid, and protein metabolism (Bindschedler *et al*., 2009). Similar profiles have been reported in *C. acutatum* (El-Akhal *et al*., 2013) and *Aspergillus nidulans* (Oh *et al*., 2010). Inspection of the classes of protein that were upregulated during the germination of resting spores revealed a higher abundance of proteins related to protein synthesis (i.e. Ef-Tu) (Table S2, Figure 4 and 5a). The dependence of conidial germination on protein synthesis has been demonstrated in *Neurospora crassa* (Mirkes, 1974), *Aspergillus nidulans* (Osherov and May, 2000) and *Botrytis cinerea* (González-Rodríguez *et al*., 2015). These studies are consistent with our results since we have found EF-Tu as the most upregulated protein, suggesting that increased protein synthesis is required in the early stages of *S. subterranea* spore germination. Protein synthesis is one of the most energy-consuming processes in both eukaryotic and prokaryotic cells (Browne and Proud, 2002). Proteins that mediate translation, such as the elongation factor Tu which is a universally conserved GTPase, are observed in the proteomes of many organisms (Macek *et al*., 2008; Pereira *et al*., 2015).

Our results showed that both protein synthesis and transcription occurs during the germination of *S. subterranea* and are probably required for its progression (Zhou *et al*., 2019). The expression levels of both histone H2A and histone H1 were increased in response to germination stimulants. It has been demonstrated that sporulation defects occur in strains when the expression level of H2A/ H2B-encoding genes is reduced (Norris and Osley, 1987; Tsui *et al*., 1997). The lower amount of H2A/H2B can also prevent proper regulation of the transcription cascade (Govin and Berger, 2009). The upregulation of proteasome-associated

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proteins (proteasome subunit alpha type) provides further evidence for the importance of transcription during spore germination in *S. subterranea.* The upregulation of this protein has been reported in the germination of *Nosema bombycis* spores (Liu *et al*., 2016) and might indicate a large turnover of proteins associated with the germination of resting spores (Seong *et al*., 2008) Wang *et al*. (2011) showed that proteasome inhibitors can affect the pathogenicity of *Magnaporthe oryzae*, the rice blast fungus, and delayed its spore formation as well.

Another interesting protein that showed a significant change in our study was DJ-1_PfpI domain-containing protein which was upregulated during the germination of *S. subterranea* resting spores (Figure 4, 6a and Table S2). DJ-1 domain-containing proteins, known as "DJ-1" or heat-shock proteins (Hsp proteins), are multifunctional proteins that have been reported to be involved in the regulation of transcription and mitochondrial function and having protease and molecular chaperone activities (Zhao *et al*., 2014). Abdallah *et al*. (2016) showed that YhbO and YajL, members of the PfpI/Hsp31/DJ-1 superfamily, are involved in protection against environmental stresses and like DJ-1 and Hsp31, they repair methylglyoxal-glycated proteins. In *Saccharomyces cerevisiae*, DJ-1 family members regulate mitochondrial homeostasis, cell cycle and apoptosis (Bankapalli *et al*., 2020). In *Escherichia coli*, these proteins are shown to perform multiple functions, including acid resistance and holdase chaperone activity (Subedi *et al*., 2011). However, the underlying molecular mechanism involved in cellular processes by DJ-1 domain-containing proteins is poorly understood. The upregulation of DJ-1_PfpI domain-containing protein during the germination of *S. subterranea* is probably related to its role in the regulation of transcription (Zhao *et al*., 2014). A noteworthy finding is that most of the upregulated proteins were involved in the regulation of transcription and RNA synthesis which raises the possibility that

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the source of the transcripts required for translation during the germination of *S. subterranea* resting spores could be derived from newly synthesized mRNA (Segev *et al*., 2012).

Some major structural proteins have been characterized in this study. We found that both actin and beta-tubulin proteins were significantly downregulated during *S. subterranea* spore germination (Figure 4 and 6b). It has been shown that the distribution of actin patches polarize and depolarize in the germinating spore in *S. cerevisiae* (Kono *et al*., 2005). Similarly, Horio and Oakley (2005) demonstrated the important role of tubulin in the hyphal tip growth of *A. nidulans*. The polarization of actin patches which happen in the early stage of spore germination, maintain their localization to the site of cell surface growth. In line with the above studies, our results indicated that the changes in the actin cytoskeleton are necessary for the morphological changes during the germination of the resting spore in *S. subterranea*. However, not enough is known about *S. subterranea* cell wall composition and further experimental evidence will be required to determine how actin and beta-tubulin are organised within the cell wall.

In spite of the fact that similar to the other biotrophic plant pathogens, *S. subterranea* is reduced in several metabolic pathways (Schwelm *et al*., 2015), our results indicated a significant change in metabolic pathways during the germination of *S. subterranea* resting spores (Figure 5). We also identified several proteins that showed expression in non-germinating spores but were absent in the germinating spores (Figure S2 and Table S3). Argininosuccinate synthase is one of the non-germination-specific proteins identified in the present study. This enzyme participates in metabolic pathways that are linked to the varied uses of the amino acid arginine. The phosphorylation/dephosphorylation of arginine is shown to be related to spore germination in bacteria (Zhou *et al*., 2019). In fact, arginine phosphomodification can mediate the degradation of spore proteins that need to be removed to enable germination (Trentini *et al.*, 2016). Acetyl-coenzyme A synthetase, a key molecule

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that participates in many biochemical reactions in carbohydrate, lipid, amino acid, and protein metabolism, is another non-germination-specific candidate protein. In *Colletotrichum higginsianum*, acetyl-coenzyme A synthetase gene（*ChAcs*) has been shown to be essential for carbon utilization, lipid metabolism and virulence (Gu *et al*., 2019). Before germination, during the dormant phase, the pathogens must utilize nutrient sources present in the spore, such as lipids, trehalose and glycogen (Wilson and Talbot, 2009). Both glycogen and trehalose break down into glucose and then to pyruvate. In mitochondria, pyruvate is converted into acetyl-coenzyme A, which is required for the tricarboxylic acid (TCA) cycle (Salazar *et al*., 2009).

In conclusion, our findings here represent a significant advance in our understanding of the germination of *S. subterranea* resting spores. We have revealed that protein and RNA synthesis occurred during the germination of spores in *S. subterranea*. We have also identified several candidates for resting spore- and germination- specific proteins that are likely to take part in the maintenance of dormancy of resting spores as well as the virulence of *S. subterranea*. The upregulation of the multifunctional protein DJ-1 is one of the major findings of the present experiment. However, how this protein is involved in the subsequent steps of spore germination is another important question that should be addressed in future studies. Not only helping to gain a better understanding of the germination process of *S. subterranea*, the work presented here is also a steppingstone on the way to further study of the biology of other obligate biotrophic pathogens.

Experimental Procedures

Sporosori collection and purification

Spongospora subterranea sporosori were excised from powdery scab infected potato tubers, collected from commercial fields in Devonport, Tasmania, Australia. The infected tubers

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were first washed with running tap water, rinsed in sterile water, and then air-dried for 3 days. All scab lesions were carefully excised using a scalpel and dried at 40 °C for 3 days. The sporosori were further purified using Ludox® (HS-40 colloidal silica, Sigma, NSW, Australia) density gradient centrifugation as previously described (Balotf *et al*., 2020). Briefly, 100 mg of dried *S. subterranea* sporosori were macerated in 3 mL sterile water and filtered through two layers of cheesecloth. Two mL of filtrate was layered onto 9 mL of Ludox®, 2 mL distilled water added on the top and centrifuged at 4200 ×g for 15 min. Three mL of the uppermost band including *S. subterranea* sporosori was diluted with 40 mL of sterile water and centrifuged as before for 8 min. The resultant pellet was used for protein extraction. PCR was performed with primer pairs targeted to the 18S rRNA gene to confirm the presence of *S. subterranea* in purified samples following the method of (Hernandez Maldonado *et al*., 2013).

Resting spore germination and protein extraction

Sporosori was purified from 100 mg of dried lesions suspended in 1 mL of Hoagland's solution (Balendres *et al*., 2018) and incubated at 25 °C in the dark to induce the germination of resting spores. Sporosori samples incubated at 25 °C in the dark in absence of a germination stimulant served as the non-germination treatment. Sub-samples from stimuli-treated sporosori samples were taken daily and observed microscopically (200 - 400 X) for the presence of active zoospores. The sporosori were harvested for protein extraction when zoospores were detected in all four replicates. Purified sporosori from germination and non-germination treatments were separately lysed with 250 µL of SDS lysis buffer (5% SDS and 50 mM ammonium bicarbonate) and 25 µL of protease inhibitor cocktail (cOmplete Mini EDTA-free; Roche Diagnostics, NSW, Australia) using PowerBead tubes, ceramic 2.8 mm (Qiagen, Hilden, Germany). The mixture was homogenised three times for 60 s each using a

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Fast Prep-24 bead beater (Mp Biomedicals, Seven Hills, NSW, Australia) at 4000 ×g at room temperature with 30 s intervals between runs. Lysates were then clarified by centrifugation at 16000 ×g for 10 min. To the collected supernatant, 6 volumes of ice-cold acetone was added, and tubes incubated at -20 °C overnight to precipitate total proteins. Following incubation, the tubes were centrifuged at 16000 ×g for 10 min and pellets were washed three times with chilled acetone. The protein pellets were then resuspended in 50 µL of SDS solubilization buffer containing protease inhibitor. The experiment was performed with four independent biological replicates with the treatment groups.

Trypsin digestion via the S-Trap filters and off-line desalting

Protein samples were quantified using the Pierce™ 660nm protein assay reagent containing ionic detergent compatibility reagent (Thermo Fisher Scientific, MA, USA). 50 µg of total protein was reduced by adding 20 mM dithiothreitol, boiled at 95 °C for 10 min, cooled to room temperature and alkylated with 40 mM iodoacetamide in the dark for 30 min. Samples were then prepared according to the S-Trap microcolumns (Protifi, Farmingdale, N.Y., USA) manufacturer's instructions. In brief, samples were acidified with a final concentration of 1.2% phosphoric acid. To the samples was added six volumes of binding buffer (100 mM ammonium bicarbonate in 90 % aqueous methanol, pH 7.1) and each sample loaded onto an S-Trap micro spin column after gentle mixing. S-Trap tubes were spun at 4000 rpm for 1 min and washed three times with 150 µL of S-Trap buffer. Samples were then digested with trypsin (2 µg of sequencing-grade trypsin in 20 µL of 50 mM ammonium bicarbonate) for 1 h at 47 °C. The digested peptides were eluted using 50 mM ammonium bicarbonate and then 0.2% formic acid. Samples were further cleaned by off-line desalting using Millipore ZipTips (Merck, Darmstadt, Germany) according to the manufacturer's instructions and dried

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peptides were reconstituted in HPLC loading buffer (2% acetonitrile and 0.05% TFA in water).

LC-MS/MS analysis

Peptides (1 µg of each digest) were separated and analysed using an Ultimate 3000 nano RSLC system (Thermo Fisher Scientific, MA, USA). Tryptic peptides were first concentrated on a PepMap 100 C18 trapping column (particle size, 3 µm; length, 20 mm; diameter, 75 µm) for 5 min. Peptides were then separated using a PepMap 100 C18 analytical column (particle size, 2 µm; length, 250 mm; diameter, 75 µm) at a flow rate of 300 nL/min and held at 45 °C. A 90-minute gradient from 98% mobile phase A (0.1% formic acid in water) to 50% mobile phase B (0.08% formic acid in 80% acetonitrile and 20% water) comprised the following steps: 2-10% B over 12 min, 10-25% B over 48 min, 25-45% B over 10 min, holding at 95% B for 5 min then re-equilibration in 2% B for 15 min. The nanoHPLC system was coupled to a Q-Exactive HF mass spectrometer equipped with nanospray Flex ion source (Thermo Fisher Scientific, MA, USA) and controlled using Xcalibur 4.1 software. Spray voltage was set to 2.0 kV, S-lens RF level to 50, and heated capillary set at 250 °C. MS scans were acquired from 370-1500 m/z at 60,000 resolution, with an AGC target of 3×10^6 and a maximum fill time of 100 ms. Fragment ion scans were acquired at 15,000 resolution (scan range 200-2000 m/z), with an AGC target of 2×10^5 and a maximum fill time of 28 ms. An isolation width of 1.4 m/z was used, and normalized collision energy for HCD set to 27. MS/MS spectra were acquired in data-dependent mode using a Top15 method with 30-second dynamic exclusion of fragmented peptides. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol *et al.*, 2019) partner repository with the dataset identifier PXD022089 (Username: reviewer_pxd022089@ebi.ac.uk; Password: fT5h435B).

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Protein identification and analysis of abundance data

The MS/MS raw data were imported into MaxQuant software (v. 1.6.0.16, www.maxquant.org) for label-free quantitative comparison between germinating and resting spores. Searches were conducted using the Andromeda search engine with default search settings against the *S. subterranea* database downloaded from UniProt (www.uniprot.org/proteomes/,containing 11,129 proteins). Searches were restricted to a peptide false discovery rate (FDR) of 1% for both peptide-spectrum matches and protein identification. The match-between-runs function was enabled. Statistical analysis of LFQ data was performed by LFQ Analyst (Shah *et al*., 2019) and the Perseus software (v. 1.5.0.15, www.perseus-framework.org). Protein groups identified either as potential contaminants, by reverse database matching or identified "only by site" were removed. The default data processing workflow used in LFQ-Analyst only retains proteins that have 75% or more valid values and identified based on at least two unique peptides. Missing values were imputed using the normal distribution of protein abundances left-shifted by 1.8 standard deviation with a width of 0.3. A Benjamini–Hochberg test (Benjamini and Hochberg, 1995) was used to identify the differentially abundant proteins between germinating spores and control samples. Proteins with a fold-change of 1.3 times or more and the FDR of 0.05 or less were defined as quantitatively significant. The proteinGroups.txt output file is presented in Supplemental Tables S4.

Annotation and Gene Ontology (GO) Enrichment Analysis

The protein sequences of each accession number in *S. subterranea* proteome were obtained using the Basic Local Alignment Search Tool (BLAST). The functional categorization was obtained from the GO terms of each protein according to the UniProt database (www.uniprot.org) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) entries

(www.genome.jp/kegg/). Enzyme Commission (EC) was retrieved from the Expasy database (https://enzyme.expasy.org/). Heatmap was drawn using Perseus software and volcano plot was obtained from LFQ Analyst platform (https://bioinformatics.erc.monash.edu/apps/LFQ-Analyst/).

RNA extraction and real-time PCR analysis

To validate the proteomics data, a quantitative real-time PCR (qRT-PCR) analysis was used to measure the mRNA transcript level for the six randomly selected DEPs. For each sample, 100 mg of dried *S. subterranea* sporosori were purified using Ludox® density gradient centrifugation. Half the samples were suspended in 1 mL of Hoagland's solution to stimulate the germination of the spores. Spores were collected 4 days after treatment when motile zoospores were observed in the germinating samples. Two hundred μL of an overnight culture of the marine bacterium *Pseudoalteromonas prydzensis* was added to each sample and used as internal control to confirm RNA quality and to normalise qPCR data. Total RNA from the samples was isolated using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The amount of RNA was determined using a Qubit™ RNA BR Assay Kit (Invitrogen, Waltham, MA). Total RNA was then treated with the DNase I (Qiagen, Hilden, Germany) to remove any remaining DNA from the samples before cDNA synthesis. Primers were designed using the Primer3 (Version 4; www.bioinfo.ut.ee/primer3-0.4.0/) and NCBI Primer-Blast (www.ncbi.nlm.nih. gov /tools/primer-blast). Quantitative real-time PCR experiments were carried out in 20 μL volume in a Qiagen RotorGeneQ (Qiagen, Hilden, Germany) using iTaq Universal SYBR Green Supermix (Bio-Rad, NSW, Australia). Data were normalized using the internal control and the fold change of each gene was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The quantitative gene

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expression analysis were performed with three biological replicates and three technical replicates. The primer sequences were listed in Table S5.

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Figure legends

Figure 1. (a) Powdery scab lesions on potato tubers and (b) agarose gel electrophoresis of the PCR product of 18S rRNA gene for DNA extracted from purified powdery scab materials. Lane M: MassRuler DNA ladder; lane NTC: non-templet control; lane 1 & 2: showing amplified *S. subterranea* 18S rRNA gene in purified samples.

Figure 2. Distribution of identified proteins from the proteome of *S. subterranea* into gene ontology (GO) categories, according to their involvement in biological processes (right) and their molecular function (left).

Figure 3. (a) Pathways analysis and (b) enzyme classes of *S. subterranea* identified proteins.

Figure 4. Comparison of differently abundant proteins in germinating and resting spores of *S. subterranea*. Proteins with FDR < 0.05 are indicated by dark data points and labeled with their respective accession numbers, details of which are provided in Table S2. N-GS: Non-germinating Spores; GS: Germinating Spores.

Figure 5. GO enrichment analysis of (a) the upregulated and (b) downregulated proteins during the germination of *S. subterranea* spores. MF: molecular function; CC: cellular components; BP: biological process.

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Figure 6. Transcript levels of genes encoding (a) upregulated and (b) downregulated proteins. Error bars represent the standard deviation based on three independent biological and three technical replicates. Asterisk (*): significant mRNA difference between germinating and resting spores ($p < 0.05$); 'ns': no significant difference ($p \geq 0.05$); N-GS: Non-germinating Spores; GS: Germinating Spores.

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