Brief Report

Scavenging organic nitrogen and remodelling lipid metabolism are key survival strategies adopted by the endophytic fungi, *Serendipita vermifera* and *Serendipita bescii* to alleviate nitrogen and phosphorous starvation in vitro

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Summary

*Serendipitaceae* represents a diverse fungal group in the Basidiomycota that includes endophytes and lineages that repeatedly evolved ericoid, orchid and ectomycorrhizal lifestyle. Plants rely upon both nitrogen and phosphorous, for essential growth processes, and are often provided by mycorrhizal fungi. In this study, we investigated the cellular proteome of *Serendipita vermifera* MAFF305830 and closely related *Serendipita vermifera* subsp. *bescii* NFPB0129 grown in vitro under (N) ammonium and (P) phosphate starvation conditions. Mycelial growth pattern was documented under these conditions to correlate growth-specific responses to nutrient starvation. We found that N-starvation accelerated hyphal radial growth, whereas P-starvation accelerated hyphal branching. Additionally, P-starvation triggers an integrated starvation response leading to remodelling of lipid metabolism. Higher abundance of an ammonium transporter known to serve as both an ammonium sensor and stimulator of hyphal growth was detected under N-starvation. Additionally, N-starvation led to strong up-regulation of nitrate, amino acid, peptide, and urea transporters, along with several proteins predicted to have peptidase activity. Taken together, our finding suggests *S. bescii* and *S. vermifera* have the metabolic capacity for nitrogen assimilation from organic forms of N compounds. We hypothesize that the nitrogen metabolite repression is a key regulator of such organic N assimilation.

Introduction

Mycorrhizal symbioses are ubiquitous associations occurring between soil fungi and the root systems of almost all plant species (Ray *et al.*, 2005; Smith and Read, 2008; Bonfante and Genre, 2010). In exchange for photosynthetically-derived carbon, plants rely heavily on their mycorrhizal partners for macro and micronutrient acquisition and transfer, especially in marginal soil types (Cardoso and Kuyper, 2006). As a consequence, studies on these symbioses have largely focused on growing both plant and symbiont on replete media or in media lacking certain soil nutrients, such as nitrogen (N) and phosphorous (P), and evaluating the shift in protein abundance or gene expression. Ectomycorrhizal and arbuscular mycorrhizal fungi have been studied extensively for nutrient transfer to plants, owing both to their ecological and economic importance as well as their phylogenetic distribution and the number of plant and fungal species involved (Heijden *et al.*, 2015). The *Serendipitaceae* (formerly Sebacinales Group B) (Oberwinkler *et al.*, 2014) represents a diverse group of fungi in the Basidiomycota that encompasses endophytes and lineages that repeatedly evolved ericoid, orchid and ectomycorrhizal abilities (Weiss *et al.*, 2016). They often provide N and P to their host plant (*Sherameti* *et al.*, 2005; *Yadav* *et al.*, 2010), particularly in the nutrient-limiting soils (Nurfadilah *et al.*, 2013). Hence, similar study with *Serendipitaceae* fungi is warranted.
Serendipita vermifera MAFF 305830 (= Sebacina vermifera) was first isolated from an Australian orchid, Cyrtostylis reniformis (Warcup, 1988). Previous research performed in our laboratory with this strain of Serendipita vermifera indicates its plant growth promoting abilities in a variety of plants (Ghimire and Craven, 2011; Ray et al., 2015; Ray and Craven, 2016), where little or no host specificity is found. Considering their proven beneficial impact on plant growth and their ubiquity, we describe Serendipitaceae as an inconspicuous, but amenable and effective microbial tool for enhancing plant productivity and stress tolerance. Unfortunately, the agronomic utility of these fungi is hampered by the paucity of strains available, the large majority isolated from Australian orchids. We have addressed this constraint by isolating the first North American strain of Serendipita, named Serendipita vermifera subsp. bescii NFPB0129 (hereafter referred to as S. bescii), from the roots of a switchgrass plant in Ardmore, Oklahoma (Craven and Ray, 2017; Ray et al., 2018).

The ability of Serendipitaceae to support plant nutrition is still a matter of debate, because there are contrasting reports on this question (Ngwene et al., 2016). Thus, in order to understand how Serendipitaceae fungi can provide N and P to their host plant, it is at first important to elucidate their own nutrient uptake capacities. Here, we sought to characterize the proteome of S. vermifera and S. bescii under N and P starvation conditions in vitro. The overall objective of the study was to elucidate the cellular response(s) of two Serendipitaceae strains towards stress imposed by the lack of one or the other of these important nutrients. Our approach allows not only to evaluate N and P acquisition processes in the understudied Serendipitaceae, but also to understand the physiological responses to their restriction. Although cultures of these fungi are severely limited at this point, two strains from North America and Australia were compared, enabling a crude cross-taxon proteomic comparison. This research represents the first step towards a holistic model of N and P metabolism in Serendipitaceae.

Results and discussion

Effect of N or P deficiency on growth and colony morphology of S. vermifera and S. bescii

To assess the impact of N or P starvation on growth and colony morphology of these fungi, the two strains were grown in MMN agar plates and in liquid culture, under optimum, N (ammonium)-starved and P (phosphate)-starved conditions for 14 days (Fig. 1A and B). On MMN agar plates, S. vermifera and S. bescii exhibited a similar growth pattern. Radial growth in terms of colony diameter was the highest in the N-starved condition (Figs. 1[i], and 2). In contrast, hyphal density, reflected in the prevalence of hyphal branching, was maximized in the P-starved condition (Figs. 1[ii, iii], and 2).

It has been reported in filamentous fungi that high affinity ammonium transporters are involved in mycelial proliferation (Madhani and Fink, 1998) and may also act as ammonium sensors (Javelle et al., 2003), which stimulate hyphal proliferation in response to low levels of ammonium enrichment. Intriguingly, in our cellular proteome data we detected a putative high affinity ammonium transporter in S. bescii (Protein I.D. # 779628) (Supporting Information Table S1, sheet 1) and its orthologue in S. vermifera (Protein I.D. # 325657) (Supporting Information Table S1, sheet 3) that are highly (>12 fold) (Supporting Information Table S1, sheet 1, sheet 3) abundant under the ammonium starvation condition (Table 1). Additionally, the orthologue of this high-affinity ammonium transporter gene has already been reported to function as nitrogen sensor during nitrogen starvation conditions in Serendipita indica (PIAMT1 Protein I.D. # 72831) (Lahrmanh et al., 2013). Phenotypic observation along with the experimental proteome data suggest a role of this ammonium transporter in hyphal proliferation and nitrogen sensing in Serendipitaceae.

Increased root branching and root hair formation is an effective and well-documented strategy employed by plants to improve P (phosphate) acquisition, especially in P deficient conditions (Raghothama et al., 2005; Niu et al., 2012). In our study, a similar phenotype was observed in the colony morphology of these two fungi under a P-starved condition. Like roots in plants, hyphal branching was substantially increased, but overall fungal radial growth (i.e., analogous to primary root elongation) decreased with respect to the control (Fig. 1[i, ii and iii]).

Identification and relative abundance of proteins in S. bescii and S. vermifera whole-cell fraction

In total, 4559 and 5125 proteins were identified in S. bescii and S. vermifera whole-cell fractions, respectively, representing 32% and 33% of the total predicted protein coding genes (Fig. 3). Out of 4559 cellular proteins in S. bescii, 4034 (88%) were found to be the part of a core cellular proteome present in all conditions tested (N and P starvation, control; Fig. 3), while 87 (1.9%) and 3 (0.1%) were specific to N- or P-starvation, respectively. A similar pattern was observed in S. vermifera as well, where out of 5125 total cellular proteins, 4603 (90%) were found to be common in all tested conditions, whereas 109 (2%) and 8 (0.2%) were found to be specific to N- or P-starvation, respectively.

To identify proteins whose abundances are regulated in response to either nutrient-depleted condition, we performed pairwise comparisons and only considered proteins having an absolute value of Log2 fold-change difference >1 (i.e., two-fold change), and p-value <0.05.
as biologically significant. Using these criteria, we identified 1410 differentially abundant proteins in *S. bescii* (Supporting Information Table S1, sheet 1), in which 583 were up-regulated and 827 were down-regulated, and 1757 differentially abundant proteins in *S. vermifera* (Supporting Information Table S1, sheet 3), of which 583 were up-regulated and 827 were down-regulated.

Fig. 1. N or P starvation regulates colony morphology of (A) *S. bescii* and (B) *S. vermifera*, respectively. N and P starvation were imposed by growing the fungi in Modified Melin Norkan’s (MMN) media without N (ammonium) or P (phosphate). The same isolates were grown in MMN agar fully supplemented with N (~120 ppm) and P (~650 ppm) as controls for direct comparison. The experiment was conducted with three biological replicates for each treatment. Bar on petridish = 5 mm; Fig [ii] = 200 μm; Fig [iii] = 50 μm.

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694 were up-regulated and 1063 were down-regulated, respectively, under N starvation condition. Similarly, under P-starvation, S. bescii (Supporting Information Table S1, sheet 2), expressed 990 differentially abundant cellular proteins, of which 16 were up-regulated and 974 were down-regulated, and 327 differentially abundant proteins in S. vermifera (Supporting Information Table S1, sheet 4) under the same condition, of which 113 were up-regulated and 214 were down-regulated.

The raw spectrum files, peak list files and result files have been made available using the following ProteomeXchange accession the PRIDE (Vizcaíno et al., 2015) partner repository with the data set identifier PXD010995 and 10.6019/ PXD010995. The genome portal for S. vermifera and S. bescii can be accessed at https://genomed.jgi.doe.gov/ Sebve1/Sebve1.home.html, and at https://genomed.jgi.doe. gov/Sebvebe1/Sebvebe1.home.html, respectively.

**Effect of N or P starvation on cellular proteome in Serendipitaceae**

Serendipitaceae have metabolic capacity for nitrogen assimilation from organic forms. Under ammonium-sufficient conditions, ammonium uptake is followed by its incorporation into the key N donor glutamine. In the present study, under ammonium-starved condition, a nitrate reductase (Nr) and a nitrite reductase (Nir), all synergistically involved in N assimilation via the nitrate reductase pathway, were differentially upregulated in S. bescii and S. vermifera, respectively (Fig. 5; Supporting Information Table S1). Intriguingly, this is in sharp contrast with Serendipita indica, which lack genes for nitrogen metabolism (Oberwinkler et al., 2013) via the nitrate reductase pathway (Zuccaro et al., 2011). Additionally, nitrate assimilation system has also been reported to be absent in related orchid mycorrhizal fungi Tulasnella calospora (Fochi et al., 2017).

We performed GO (gene ontology) enrichment analysis to functionally categorize differentially abundant proteins based on their responsiveness to N or P starvation. Figure 4 represents the functional categorization of S. bescii and S. vermifera proteins that were differentially regulated due to N and P starvation, respectively.

The GO enrichment analysis of the cellular proteome in S. bescii (Fig. 4) revealed that those with peptidase activity were over-represented among the category of proteins that were more abundant due to N starvation. Additionally, proteins with carboxypeptidase activity and α-glucosidase activity were also significantly enriched in the differentially abundant proteins that were found in greater abundance due to N starvation. In particular, several proteins with hydrolase activity specifically acting on carbon-nitrogen (but not peptide) bonds were also differentially up-regulated due to N starvation, perhaps suggesting a role in organic N acquisition.

Among the proteins that were identified as more abundant due to P starvation, proteins with ubiquitin-like protein transferase activity were identified to be the largest group. The GO enrichment analysis of S. bescii proteins that were more abundant due to P starvation could not be computed given the inadequate number of proteins.

The functional categorization of S. vermifera proteins (Fig. 4) that were differentially regulated due to N starvation included proteins with anion transport activity, which were identified to be the largest group that were more abundant under N starvation, whereas proteins involved in organo-nitrogen compound biosynthesis represented the largest functional group that were less abundant due to N starvation. As with S. bescii, the GO enrichment analysis of S. vermifera proteins that were identified as more abundant due to P starvation may not be accurate due to inadequate number of such proteins.

**GO enrichment analysis of differentially abundant proteins in whole-cell fractions**

We performed GO (gene ontology) enrichment analysis to functionally categorize differentially abundant proteins based on their responsiveness to N or P starvation. Figure 4 represents the functional categorization of S. bescii and S. vermifera proteins that were differentially regulated due to N and P starvation, respectively.

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Table 1. List of selected genes that plays critical role in N or P metabolism in Serendipitaceae.

| Strain   | Protein ID | Protein description           | Regulation | Role                                      |
|----------|------------|-------------------------------|------------|-------------------------------------------|
| S. bescii | 779628     | Ammonium transporter          | Up         | Hyphal proliferation                       |
| S. vermifera | 738862 | Nitrate reductase              | Up         | NH₄⁺ assimilation by nitrate reductase pathway |
| S. bescii  | 788097     | Nitrite reductase              | Up         |                                           |
| S. vermifera | 327362 | Nitrate transporter            | Up         | Assimilation of less preferred low        |
| S. bescii  | 707892     | Oligopeptide transporter       | Up         |                                           |
| S. bescii  | 777449     | Urea transporter               | Up         |                                           |
| S. vermifera | 228455 | Ammonium transporter           | Up         |                                           |
| S. bescii  | 692622     | Urease: UreD                   | Up         |                                           |
| S. vermifera | 60481    |                                  | Up         |                                           |
| S. bescii  | 136136     | Subtilisin: Vacuolar peptidase | Up         | Vacular hydrolysis of amino acid          |
| S. vermifera | 67502     |                                  | Up         |                                           |
| S. bescii  | 12161      | NmrA-like                      | Down       | Nitrogen metabolite repression            |
| S. bescii  | 74449      | Related to ARG82 inositol      | Down       | Phosphate (polyP) biosynthesis            |
| S. bescii  | 756905     | VCX1: Ca²⁺/H⁺ antiporter       | Down       | Control of cytosolic Ca²⁺ and K⁺          |
| S. bescii  | 617618     | NmrA-like                      | Up         | Hydrolysis of phosphorylated compound     |
| S. bescii  | 12161      | NmrA-like                      | Down       |                                           |
| P Starvation |          |                                |            |                                           |
| S. bescii  | 738645     | Dicarboxylic amino acid        | Up         | Vacular transport of amino acid           |
| S. vermifera | 750865 | Amino acid transporters        | Up         | molecular weight organic N compounds      |
| S. bescii  | 65026      |                                  | Up         |                                           |
| S. bescii  | 777449     | Urea transporter               | Up         |                                           |
| S. vermifera | 228455 | Ammonium transporter           | Up         |                                           |
| S. bescii  | 692622     | Urease: UreD                   | Up         |                                           |
| S. vermifera | 60481    |                                  | Up         |                                           |
| S. bescii  | 136136     | Subtilisin: Vacuolar peptidase | Up         | Vacular hydrolysis of amino acid          |
| S. vermifera | 67502     |                                  | Up         |                                           |
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| S. bescii  | 756905     | VCX1: Ca²⁺/H⁺ antiporter       | Down       | Control of cytosolic Ca²⁺ and K⁺          |
| S. bescii  | 617618     | NmrA-like                      | Up         | Hydrolysis of phosphorylated compound     |
| S. bescii  | 12161      | NmrA-like                      | Down       |                                           |
| P Starvation |          |                                |            |                                           |
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| S. bescii  | 756905     | VCX1: Ca²⁺/H⁺ antiporter       | Down       | Control of cytosolic Ca²⁺ and K⁺          |
| S. bescii  | 617618     | NmrA-like                      | Up         | Hydrolysis of phosphorylated compound     |
| S. bescii  | 12161      | NmrA-like                      | Down       |                                           |

Low-molecular-weight organic N compounds, such as amino acids, urea, polyamides, and small polypeptides are major contributors to soil organic N in forests and in severe N-limited ecosystems. (Bending and Read, 1995; Nygren et al., 2007). Assimilation of these organic N compound essentially involves the synergistic action of several steps, including degradation of organic N polymers by peptidase activity, followed by assimilation of released peptides, oligopeptides and amino acids by specific transporters, and finally metabolism of assimilated amino acids (Shah et al., 2013) (Fig. 5). In our study, a nitrate transporter (NRT) was found to be highly (>12 fold) up-regulated under ammonium starvation conditions in S. bescii (Table 1).

Furthermore, four amino acid transporters and five oligopeptide transporters were significantly up-regulated under N-starvation conditions in S. bescii. Likewise, eight amino acid transporters were found, which were significantly up-regulated in S. vermifera. These transporters are presumably involved in assimilation of less preferred low-molecular-weight organic N compounds. Additionally we detected a urea transporter gene DUR3 in S. bescii (Protein I.D. # 777449) (Supporting Information Table S1) and its orthologue in S. vermifera (Protein I.D. # 228455) (Supporting Information Table S1) that were highly (>12 fold) up-regulated under ammonium-starvation conditions. Additionally, one urease accessory protein UreD, which facilitates hydrolysis of urea to ammonium, was also up-regulated under N starvation conditions in both S. bescii and S. vermifera (Table 1). This indicates that at least some fungi in the Serendipitaceae possess the enzymatic capacity to assimilate ammonium from urea under ammonium-starved conditions.

The assimilated ammonium is eventually converted into the amino acid glutamine and localized into vacuoles. DIP5 (Dicarboxylic amino acid permease), which facilitates vacuolar transport of glutamine was found to be highly abundant in both S. vermifera and S. bescii, respectively, under N-starvation conditions. However, only a small portion of assimilated amino acids is used for protein synthesis. The rest are compartmentalized into vacuoles and subsequently hydrolyzed by vacuolar subtilisin and carboxypeptidase. In S. vermifera one vacuolar amino acid transporter was found to be differentially up-regulated under N-starvation condition. In S. bescii, six genes putatively involved in subtilase activity were also up-regulated during N starvation. Likewise, five proteins putatively involved in subtilase activity were also up-regulated in S. vermifera. Proteins with carboxypeptidase activity were also significantly enriched in the population of differentially abundant proteins that were up-regulated due to N starvation in S. bescii.

In the absence of ammonium, the process of nitrogen assimilation from less preferred sources, such as nitrate, urea, peptides, and amino acids, are regulated by nitrogen
metabolite repression (Tudzynski, 2014). In ascomycota, nitrogen metabolite repression is controlled by a GATA transcription factor (Caddick, 1992), which is under the regulation of a co-repressor NmrA (Fu et al., 1988; Andrianopoulos et al., 1998). Additionally, bZIP transcription factors have been also shown to be involved. In S. bescii, one GATA transcription factor and one bZIP transcription factor were down-regulated and six NmrA-like genes were differentially regulated (four up, two down) under ammonium-starved conditions. Consistent with the protein expression pattern in S. bescii, one GATA transcription factor was up-regulated, and one bZIP transcription factor was down-regulated and seven NmrA-like genes were found to be differentially regulated under ammonium-starved conditions in S. vermifera. To conclude, the synergistic expression of peptidases, transporters, and enzymes involved in the assimilation and metabolism of released peptides and amino acids observed in this study suggest that S. bescii and S. vermifera has the metabolic capacity to assimilate nitrogen from organic forms of N-containing compounds (Fig. 5).

P-starvation triggers remodelling of lipid metabolism in Serendipitaceae. Once absorbed by the fungus, phosphate is incorporated into the active P pool, which includes phosphorylated primary metabolites, structural molecules and nucleic acids necessary for fungal growth and primary metabolism (Ezawa et al., 2002). The surplus phosphate is taken into the cytosol and rapidly polymerized into polyphosphate (polyP) before compartmentalized in the vacuoles as the storage P pool (Klionsky (polyP) before compartmentalized in the vacuoles as the cytosol and rapidly polymerized into polyphosphate until recently. ARG82 is an inositol polyphosphate multikinase that has been identified to be responsible for polyP biosynthesis in Saccharomyces cerevisiae. In our study, we detected one inositol polyphosphate kinase related to yeast ARG82, and an additional six ATPases that were differentially down-regulated under P-starvation conditions in S. bescii (Supporting Information Table S1, Sheet 2). During the vacuolar compartmentalization of phosphate (PO$_4^{3-}$), different cations (e.g., K$^+$, Ca$^{2+}$, Mg$^+$) are also imported synchronously to neutralize the negative charge of polyP accumulation (Fig. 5). In this context, we found five antiporter proteins in S. bescii, which are predicted to be responsible for inorganic cation transport, were down-regulated under P starvation. One is predicted K$^+$/H$^+$ type antiporter responsible for cation extrusion across plasma membrane and four are predicted Ca$^{2+}$/H$^+$ antiporter (VCX1) involved in the control of cytosolic Ca$^{2+}$ and K$^+$ concentrations.

Remodelling of lipid biosynthesis through degradation of phospholipids to release phosphate is a critical P-starvation response adapted by plants aimed at improving phosphate uptake efficiency. This degradation of phospholipids is mediated by synergistic action of phospholipases and phosphohydrolases. P starvation in S. bescii resulted in an asymmetric model of distribution of differentially abundant proteins. Out of 990 differentially abundant proteins, 974 proteins were down-regulated, whereas only 16 proteins were up-regulated. We hypothesize that P starvation leads to dramatic remodelling of phosphate metabolism in S. bescii, resulting in degradation of phosphorylated metabolites, as well as phospholipids to release phosphate needed by the cell.

One of the differentially up-regulated proteins in S. bescii due to P starvation was revealed to be a protein (I.D: 690995) harbouring a pyrophosphohydrolase (NUDIX hydrolase) domain (Table 1). These pyrophosphohydrolases act upon nucleoside diphosphates, typically linked to another moiety, X (NDP-X), to yield NMP (nucleoside monophosphate) plus P-X. Substrates include (d)NTPs, nucleotide sugars...
and alcohols, dinucleoside polyphosphates, dinucleotide coenzymes and capped RNAs. A second protein, a patatin-like phospholipase, was found to be up-regulated due to P starvation in *S. bescii* (Table 1). Patatin is a phospholipase that typically catalyses the non-specific hydrolysis of phospholipids, glycolipids, sulfolipids, and mono- and diacylglycerols. In stark

Fig. 4. Overview charts of Gene ontology (GO) enrichment analysis of differentially abundant *S. bescii* and *S. vermifera* cellular proteins due to N or P starvation, respectively. Whole genome gene ontology (GO) term annotation was performed using Blast2GO (Conesa et al., 2005) with a blastp E-value hit filter of $1 \times 10^{-5}$, an annotation cutoff value of 55 and a GO weight of five. Using ClueGO (Bindea et al., 2009), observed GO biological processes were subjected to the right-sided hypergeometric enrichment test at medium network specificity selection and p-value correction was performed using the Holm-Bonferroni step-down method (Holm, 1979). GO terms that are functionally associated with one another were grouped by Kappa statistics using ClueGO and share the same colour. Each group in the pie chart is represented by the group leading GO term (i.e., the most significant term in the group). The size of the sections correlates with the number of GO terms included in each group. ‘*’ and ‘**’ indicate that the GO process is over-represented at p-value <0.05 and p-value <0.001, respectively.
contrast to \textit{S. bescii}, in \textit{S. vermifera}, a protein putatively functioning as a non-specific lipid acyl hydrolase, patatin, was significantly down-regulated under the P-starvation condition. In conclusion, we propose that lipid remodelling is a key survival strategy adopted by \textit{Serendipitaceae} fungi to alleviate P starvation.

**Acknowledgements**

\textit{Serendipita vermifera} (MAFF-305830) used in this study was obtained from the National Institute of Agro-biological Sciences, Tsukuba, Ibaraki, Japan. The authors thank Josh Meo and Fuqi Liao from Noble Research Institute for graphic design and image quantitative analysis and statistics. This work was supported by the United States Department of Energy (DOE), Office of Biological and Environmental Research (OBER) and conducted within the DOE BioEnergy Science Center (BESC) and Center for Bioenergy Innovation (CBI) projects. BESC and CBI are U.S. DOE Bioenergy Research Centers supported by OBER in the DOE Office of Science. This research used resources of the Compute and Data Environment for Science (CADES) at the Oak Ridge National Laboratory. This manuscript has been co-authored by UT-Battelle, LLC under Contract No. DE-AC05-00OR22725 with the U.S. Department of Energy.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Supplementary Table S1.** Protein abundances are represented as Log2 transformed values. Zero (i.e., null) protein abundance values were imputed with a constant value of 15, which serves as a representative of the limit of detection (LOD) for this experiment. Student’s t-tests were performed by the computational program Perseus. A protein was categorized as having a significant abundance difference between a control and nutrient starved condition if it passed the significance threshold requiring a p-value ≤0.05 and an absolute value of Log2 fold-change difference >1.