




Communication

Differential Gene Expression in the Model Actinomycete *Streptomyces coelicolor* A3(2) Supports Nitrogen Mining Dependent on the Plant Carbon to Nitrogen Ratio

Damien Finn ^{1,2,*}, Kerrilyn Catton ³, Marijke Heenan ³, Peter M. Kopittke ¹ , Diane Ouwerkerk ^{4,5} , Athol V. Klieve ^{1,5} and Ram C. Dalal ¹ 

¹ School of Agriculture and Food Sciences, The University of Queensland, Brisbane, QLD 4072, Australia; p.kopittke@uq.edu.au (P.M.K.); a.klieve@uq.edu.au (A.V.K.); r.dalal@uq.edu.au (R.C.D.)

² School of Life Sciences, Arizona State University, Tempe, AZ 85281, USA

³ Soil Chemistry Lab, Department of Science, Technology and Innovation, Queensland Government, Brisbane, QLD 4001, Australia; Kerrilyn.catton@des.qld.gov.au (K.C.); marijke.heenan@des.qld.gov.au (M.H.)

⁴ Rumen Ecology Unit, Department of Agriculture and Fisheries, Queensland Government, Brisbane, QLD 4001, Australia; Diane.Ouwerkerk@daf.qld.gov.au

⁵ Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Brisbane, QLD 4072, Australia

* Correspondence: damien.finn@uqconnect.edu.au; Tel.: +1-480-331-0302

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Abstract: Nitrogen mining is the process whereby microbial communities catabolise recalcitrant long-term organic matter (OM) to meet nutritional requirements that are not ensured by labile OM. Microbial degradation of recalcitrant OM impacts soil fertility and contributes to greenhouse gas emissions in agricultural systems. Here we conducted a transcriptomics study to track differential gene expression in the model soil Actinomycete *Streptomyces coelicolor* A3(2) during the decomposition of mung bean (*Vigna radiata* L.) and wheat (*Triticum aestivum* L.) residues of relatively low and high carbon-to-nitrogen (C:N) ratios (17.3 and 35.7, respectively) at 1, 7, and 14 days of incubation. A negative binomial general linear model showed that plant variety predominantly affected transcription ($p < 0.001$), although time of incubation also had an effect ($p = 0.01$). In the high C:N ratio treatment, the expression of cellulases, chitinase, N-acetylglucosaminidase, secreted peptidases, and mineral nitrogen (N) metabolism were increased after 24 h. The low C:N ratio treatment demonstrated preferential expression of glutamate dehydrogenase, transporters involved in glutamate uptake and glycolysis, indicating more efficient N and carbon (C) assimilation. After 14 days, the low C:N ratio treatment showed increased transcription of extracellular enzymes, glutamate dehydrogenase, and glutamate transport. These results show an important role for added plant organic N content in determining when the transcription of genes associated with N mining occurs.

Keywords: plant decomposition; soil organic matter; soil microbial function

1. Introduction

It is well-documented that the conversion of native vegetation for agricultural land use leads to losses of 25–50% of soil organic carbon (SOC) [1]. Losses of SOC and the associated nitrogen (N) and phosphorus (P) (collectively referred to here as soil organic matter or SOM) from agricultural soils threatens future food security due to decreases in crop productivity, increased soil erosion, and is a net source of atmospheric greenhouse gases [2–4]. Microbial metabolism converts input organic

matter to form stable, long-term metabolites adsorbed to soil particles, which could be a means to restore SOM in agricultural systems [5–7]. This SOM can be further metabolised (and therefore not retained) to meet cellular requirements in N-poor systems. This process is referred to as N mining, which is characterised by a net increase in decomposition as microorganisms degrade recalcitrant organic matter (OM) to acquire N [8–10]. Consequently, the carbon-to-nitrogen (C:N) ratio of input organic matter can affect the retention of SOM [8,11,12].

Bacterial taxa within the phylum Actinobacteria are often observed to be relatively more abundant in agricultural soils compared to other phyla [13–15]. Members of the order Actinomycetales, in particular, are ubiquitous within soil, water, and plant litter environments, and have long been considered to play an important role in carbon (C) cycling [16]. *Streptomyces coelicolor* A3(2) has been studied extensively by the scientific community [17] and the 8.67 Mbp linear genome of *S. coelicolor* A3(2) has been sequenced and annotated [18]. The majority of this microorganism's genome is devoted to the regulation, transportation, and degradation of extracellular nutrients, and it is well adapted to the spatially heterogeneous and temporally variable soil environment [18]. Metabolic analyses have observed sensitivity to nutrient availability, with switches from the Embden–Meyerhof–Parnas pathway to the pentose phosphate pathway when transitioning from exponential to stationary growth [19], in addition to significant changes in the regulation of N and P metabolism during this transition [20]. Both genomic and metabolic analyses support the utilisation of a variety of C sources by *S. coelicolor* A3(2), such as glucose, cellulose, cellobiose, xylose, glycerol, galactose, fructose, arabinose, chitin, and N-acetyl glucosamine [18,19]. The metabolic diversity and strict metabolic regulation of this soil microorganism make it an ideal candidate to better study N mining responses in a model system.

The objective of this study was to study differential gene expression (DGE) in *S. coelicolor* A3(2) during the decomposition of plant material with varying N concentrations. These were: (a) wheat (*Triticum aestivum* L.) (C:N ratio 35.7); and (b) mung bean (*Vigna radiata* L.) (C:N ratio 17.3). Differential gene expression was compared over time (at 1, 7, and 14 days of incubation). We hypothesised that *S. coelicolor* A3(2) would undergo significant transcriptional changes in response to plant material with varying C:N ratios, targeted at greater acquisition and catabolism of N sourced from native SOM in response to wheat, relative to mung bean.

2. Materials and Methods

2.1. Soil and Plant Sampling

Soil, an alfisol, was collected from a long-term phosphorus fertilisation trial in Horsham, Australia (−36.672547° N, 142.288467° E). A bulk soil sample was taken from the top 0–10 cm depth. Soil was homogenised and dried at 40 °C, >2 mm root material was removed, ground, and passed through a <2 mm sieve. Soil particle size analysis, Colwell-phosphorus (Colwell-P) extraction (NaHCO₃ at pH 8.2), pH, and electrical conductivity (EC) were measured as described in Reference [21], with pH and EC measured using a 1:5 soil-to-water suspension. Field capacity moisture (FCM) was determined using the container capacity method, which is equivalent to 55–75% water-filled pore space [22]. The soil texture was as follows: 48.6% sand, 33.3% silt, and 18.1% clay. The Colwell-P concentration was 22 mg kg^{−1} soil. The soil pH was 5.5. The EC was 0.1 mS dm^{−1} and the FCM was 36.5%. A soil subsample was ground to <100 µm particle diameter and total C and N was measured on a TruMac CN analyser (LECO, Castle Hill, Australia). The soil total organic carbon (TOC) was 6%, and total nitrogen (TN) was 0.48% (C:N ratio of 12.5). To homogenise the plant material, it was dried at 60 °C for 72 h prior to freezing at −17 °C for 24 h. The plant material was subsequently freeze-dried with a primary stage at 0 °C for 24 h and a secondary stage at 30 °C for three days. The dried material was ground to <100 µm particle diameter. The C and N contents of plant residues were 42.8% and 1.2% in wheat (C:N ratio of 35.7), and 38% and 2.2% in mung bean (C:N ratio of 17.3), respectively.

2.2. Bacterial Strain and Culture Conditions

S. coelicolor A3(2) was obtained from the American Type Culture Collection (ATCC), ATCC identification no.: BAA-471. The organism was cultured on ATCC medium 1877, ISP medium 1 (tryptone–yeast extract) and dextrose–starch agar (Becton–Dickinson, Franklin Lakes, NJ, USA) at 28 °C. To identify the exponential growth phase, a growth curve assay was performed at 0, 1, 3, 7, 14 and 20 days with a LIUV-201 UV/Vis spectrophotometer at 600 nm (Lambda Scientific, Edwardstone Town, Australia).

2.3. Soil Incubation

A subsample of soil was treated with gamma-irradiation to a total gamma-ray dosage of 35 kGy [23,24]. A total of 2 g of gamma-irradiated soil and 200 mg of either ground wheat or mung bean material (10% weight/weight) were added to a sterilised gas-tight Hungate tube (Bellco, Vineland, NJ, USA). Once *S. coelicolor* A3(2) had reached the beginning of the exponential growth phase, cells were washed twice with 5 mL sterile 10 mM potassium phosphate buffer (PPB) (pH 6.8) by centrifugation at $3000 \times g$ for 10 min and resuspended in 1 mL 10 mM PPB. To bring the soil to field capacity moisture, 720 microlitres of *S. coelicolor* A3(2) cells resuspended in 10 mM PPB were added to the treatments. Negative controls were prepared by adding 720 μ L 10 mM PPB to duplicate gamma-irradiated soil samples. All incubations were performed in triplicate. Final sample weights were noted to readjust moisture levels with 10 mM PPB over time. Incubations were performed statically at 28 °C.

2.4. RNA Extraction, Sequencing, and DGE Analysis

Incubated soils were resuspended in 4 mL of 10 mM PPB, aliquoted separately in 1 mL volumes, and preserved by snap-freezing on liquid nitrogen. Total RNA was extracted from 1 mL soil resuspensions at time points 1, 7, and 14 days with MoBio RNA Powersoil Total RNA Isolation kits (MoBio Laboratories, Carlsbad, CA, USA), in accordance with the manufacturer's instructions. Extracted RNA was treated with RNase-free DNase I (New England Biolabs, Ipswich, MA, USA). Messenger RNA library preparation and transcriptomic sequencing on the Illumina HiSeq High Throughput platform with 150 bp single read chemistry was performed by the Australian Genome Research Facility (AGRF, Melbourne, Australia). All statistical analyses were performed in R version 3.4.1 [25]. For DGE analyses, sequencing data was first run through Trimmomatic [26] and FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/) for quality control purposes. Bowtie2 [27] was used to align reads to the *S. coelicolor* A3(2) genome with local alignment and zero mismatches to improve the stringency of read mapping. SAMtools [28] was used to convert the output of Bowtie2 to the BAM format. In R, DESeq2 was used as described in Reference [29]. Sequence counts below 20 were filtered out. From the results of the *S. coelicolor* A3(2) DGE analyses, pairwise comparisons of C and N metabolism were conducted via Student's *t*-tests, using the plant substrate as a categorical variable. A negative binomial general linear model was used to compare the effects of the C and N metabolism genes with time and plant substrate [30]. Finally, the sequence data was archived with the European Nucleotide Archive with the Project ID: PRJEB23455.

3. Results

A transcriptomics approach centred on the metabolism of *S. coelicolor* A3(2) in gamma-irradiated soil was chosen in this study for several reasons. Firstly, shotgun metagenomics approaches in soils are plagued by both low rates of annotation and low sequencing coverage due to the enormous biodiversity inherent in soil ecosystems. Secondly, the genomics of *S. coelicolor* A3(2) have been exhaustively studied due to its industrial applications as an antibiotic producer [20], as a model organism in terms of genetic regulation [17], and as a model organism for life in soil [18]. Consequently, it has been possible to explore the DGE of potential genes associated with N mining in greater detail

than would be possible by considering the shotgun metagenomics of a complex microbial community during plant decomposition.

The total sequences per sample were 13.17 ± 1.8 million, and total number of reads passing Trimmomatic was $99.68 \pm 0.1\%$. Of the 6123 non-zero *S. coelicolor* A3(2) transcriptional reads, 755 (12.3%) demonstrated a significant log-fold increase ($p < 0.05$), and 763 (12.5%) demonstrated a significant log-fold decrease ($p < 0.05$) between treatments and over time. A collection of genes encoding for transcriptional regulators involved in nutrient sensing and membrane transport, and extracellular enzymes involved in nutrient acquisition, and core C and N metabolism were chosen for in-depth analyses (Figure 1). A negative binomial general linear model of the log-fold change in the expression of these genes showed strong differences between plants ($p < 0.001$), and to a lesser extent over time ($p = 0.01$).

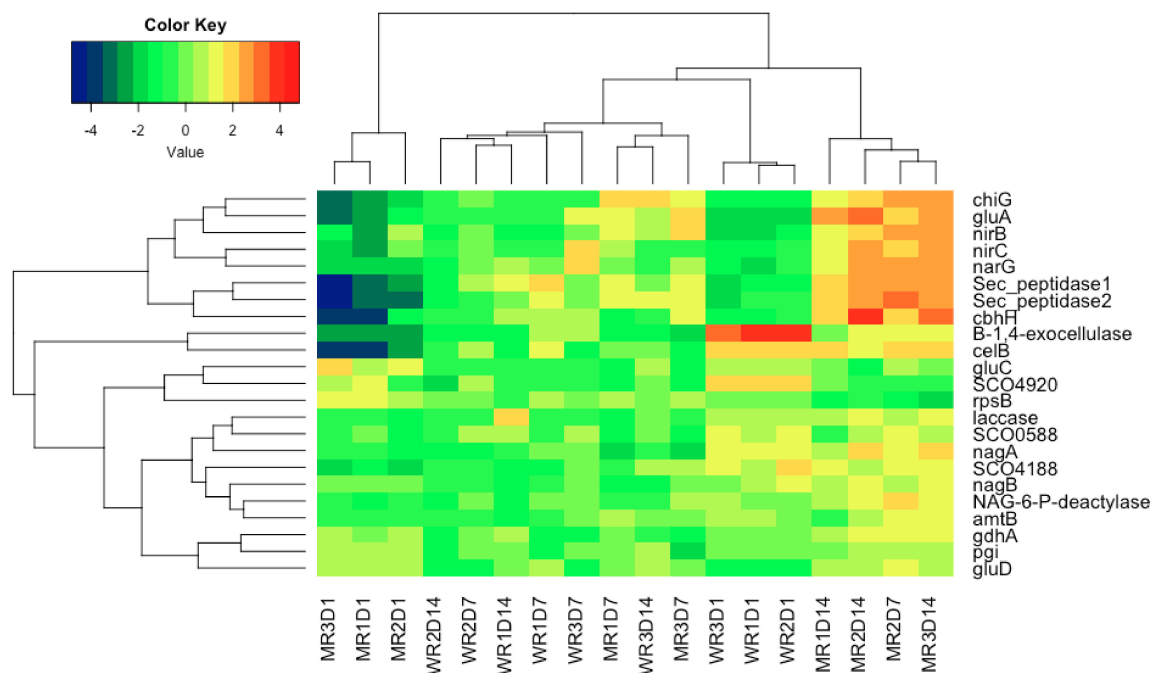


Figure 1. Heatmap of log-fold change (LFC) comparisons of differential gene expression of *S. coelicolor* A3(2) regulation, extracellular enzymes, and core C and N metabolism genes. A relative decrease in LFC is dark blue, while increases are red. Sample names are coded as follows: M (mung bean); W (wheat); R (replicates 1, 2, and 3); D (days 1, 7, and 14). Dendrograms are based on sample similarity (x-axis) and gene expression similarity (y-axis).

After 24 h of incubation, the DGE of *S. coelicolor* A3(2) was highly supportive of the N mining hypothesis. The relatively high C:N ratio of the wheat substrate elicited increased expression of a number of extracellular enzymes involved in C and N acquisition from plant material and SOM (cellulases *celB* and a putative beta-1,4-exocellulase, chitinase *chiG*, N-acetyl glucosaminidase *nagA*, a secreted peptidase, and lignin-degrading laccase) and increases in mineral N metabolism (*nirC*) and ammonium uptake from the environment via the ammonium transporter *amtB* (Table 1). The increased transcription of extracellular enzymes involved in N metabolism was reflected by the transcription of several regulators fundamental to regulating N metabolism in *S. coelicolor* A3(2), such as: (a) the upregulation of the nutrient availability sensor and CAZy regulator SCO4920 [31]; (b) the upregulation of the mineral N metabolism regulator SCO0588 [32]; and (c) the upregulation of the nutrient availability sensory and developmental regulator SCO4188 [33].

Table 1. Log fold change (LFC) comparisons of the differential gene expression of *S. coelicolor* A3(2). The mean and standard deviation of three replicates are shown for wheat and mung bean treatments. Student's *t*-tests were performed to compare treatments separately for days 1, 7, and 14. Significantly different LFCs are noted as: $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***)

Category	Gene	Day 1			Day 7			Day 14		
		Wheat	Mung Bean	Average LFC	Wheat	Mung Bean	Average LFC	Wheat	Mung Bean	Average LFC
Regulation	SCO4920 deoR-family reg	2.07 ± 0.2	0.71 ± 0.4	1.36 *	−0.15 ± 0.9	−1.05 ± 0.3	0.9	−0.61 ± 0.9	−0.46 ± 0.4	0.14
	SCO4188 gntR-family reg	1.25 ± 0.4	−1.77 ± 0.2	3.01 **	−0.63 ± 0.6	0.97 ± 0.6	1.6 *	−0.48 ± 0.9	1.25 ± 0.4	1.73
	SCO0588 sensor kinase	1 ± 0.46	−0.72 ± 0.7	1.72 *	0 ± 0.5	−1.47 ± 0.1	1.47 *	−0.02 ± 0.6	0.27 ± 0.8	0.29
Membrane	SCO5583 <i>amtB</i>	0.36 ± 0.3	−0.38 ± 0	0.74 *	−0.51 ± 0.6	0.52 ± 0.4	1.03	−0.7 ± 0.4	0.38 ± 1	1.07
	SCO2831 <i>gluA</i>	−1.8 ± 0.2	−2.39 ± 1	0.58	−0.42 ± 0.1	1.5 ± 0.4	1.92 **	−0.11 ± 0.6	2.76 ± 0.4	2.87 **
	SCO5775 <i>gluC</i>	0.69 ± 0.1	1.29 ± 0.5	0.61	−0.56 ± 0.1	−1.01 ± 0	0.45	−0.26 ± 0.5	0.67 ± 0.1	0.19
	SCO5774 <i>gluD</i>	−1.28 ± 0.1	0.67 ± 0.1	1.94 ***	−1.11 ± 0.1	0.42 ± 0.7	1.53 *	−0.31 ± 0.7	0.6 ± 0.3	0.92
Extracellular Enzymes	SCO7176 Sec peptidase1	−1.39 ± 0.4	−3.53 ± 1.3	2.14	0.8 ± 0.9	1.7 ± 0.8	0.9	−0.06 ± 1	2.48 ± 0.5	2.54 *
	SCO7188 Sec peptidase2	−1.08 ± 0.8	−3.53 ± 0.7	2.45 *	−0.44 ± 0.4	2 ± 0.8	2.44 ***	0.16 ± 1.2	2.34 ± 0.5	2.19
	SCO1187 <i>celB</i>	1.86 ± 0.2	−3.23 ± 0.7	5.1 **	1 ± 0.5	−0.73 ± 0.7	1.73 *	−0.71 ± 0.4	1.6 ± 0.3	2.3 **
	SCO6548 put beta-1,4-exocellulase	3.72 ± 0.4	−2.76 ± 0.2	6.49 ***	0.54 ± 0	−1.36 ± 0.4	1.9 ***	−1.29 ± 0.3	0.85 ± 0.8	2.14 *
	SCO7069 <i>cbhH</i>	−0.84 ± 0.3	−3.14 ± 1.4	2.29	0.46 ± 0.8	1.6 ± 0.6	1.14	−0.13 ± 0.9	2.93 ± 1.2	3.06 *
	SCO0481 <i>chiG</i>	−1.32 ± 0.3	−2.7 ± 0.4	1.38 *	−0.37 ± 0.1	1.94 ± 0.6	2.31 **	0.49 ± 1.5	1.96 ± 0.9	1.49
	SCO2758 <i>nagA</i>	1.41 ± 0.1	−0.88 ± 0.3	2.29 **	0.2 ± 0	−2.03 ± 0.1	2.23 ***	−0.89 ± 0.4	1.49 ± 0.7	2.39 *
	SCO6712 laccase	−0.74 ± 0.4	0.63 ± 0.2	1.36 *	−0.88 ± 0.1	−0.97 ± 0.5	0.09	1.1 ± 0.6	0.3 ± 1	0.8
	SCO4284 NAG-6-P deacetylase	0.27 ± 0.2	−0.92 ± 0.1	1.19 ***	0.03 ± 0.2	0.67 ± 1	0.64	−0.93 ± 0.3	0.88 ± 0.3	1.8 **
	SCO5236 <i>nagB</i>	0.88 ± 0.1	−0.02 ± 0.1	0.9 ***	−0.86 ± 0.1	0.47 ± 0.3	1.33 **	−1.04 ± 0.2	1.14 ± 0.3	2.17 **
Core Metabolism	SCO6535 <i>narG</i>	−1.34 ± 0.5	−2.01 ± 0.1	0.67	0.6 ± 1	1.03 ± 1	0.43	−0.44 ± 0.8	2.17 ± 0.5	2.61 *
	SCO2487 <i>nirB</i>	−1.9 ± 0.3	−1.01 ± 1.6	0.89	−0.59 ± 0.5	2.03 ± 0.7	2.62 **	−0.55 ± 0.9	1.72 ± 0.6	2.27 *
	SCO2488 <i>nirC</i>	−1.29 ± 0.3	−2.16 ± 0.3	0.87 *	−0.33 ± 0	1.14 ± 0.6	1.47 *	−0.44 ± 0.1	2.05 ± 0.9	2.49 *
	SCO4683 <i>gdhA</i>	−0.08 ± 0.2	0.34 ± 0.1	0.42 *	−0.94 ± 0.8	−0.57 ± 0.5	0.37	−1.06 ± 0.6	1.02 ± 0.4	2.09 *
	SCO1942 <i>pgi</i>	0.16 ± 0.1	0.61 ± 0.2	0.46 *	−0.78 ± 0.7	0.22 ± 0.1	1	−0.14 ± 1	0.38 ± 0.5	0.52
	SCO5624 <i>rpsB</i>	−0.02 ± 0.2	1.1 ± 0.1	1.08 **	0.27 ± 0.2	0.71 ± 0.2	0.44	−1.3 ± 0.6	−0.4 ± 0.1	0.9

At 24 h, the upregulated genes in the mung bean treatment were *gdhA*, *gluD*, *pgi*, and *rpsB* (Table 1). The *gdhA* gene, which encodes the nicotinamide adenine dinucleotide phosphate (NADP)-specific glutamate dehydrogenase GDH, plays a central role in cellular N assimilation [20,34]. All potential N sources, for example, mineral N, urea, N-acetyl glucosamine, amino acids, and nucleotides were processed either extra- or intracellularly to form ammonium, which was subsequently utilised in the NADPH-dependent synthesis of glutamate via GDH. Glutamate (and to a lesser extent glutamine) acted as the sole organic N donors for N assimilation by the cell, and thus represent the bottleneck for cellular N assimilation [35]. The *gluD* gene partially encodes for a glutamate permease involved in the facilitated diffusion of extracellular glutamate into the cell, where it can also be utilised for cellular N assimilation [36]. The *pgi* gene encodes for the glucose-6-phosphate isomerase, which is an essential enzyme in glycolysis. Finally, the *rpsB* gene is a universally conserved, single-copy gene essential for protein synthesis [37] and was included as a marker to compare general cell activity between treatments. Thus, despite the upregulation of *S. coelicolor* A3(2) extracellular enzymes and mineral N metabolism to acquire additional N sources in response to the high C:N ratio of wheat, the higher transcription of GDH facilitated the diffusion of extracellular glutamate into the cell. Moreover, glycolysis and higher potential protein synthesis indicate that *S. coelicolor* A3(2) was assimilating both C and N more effectively in the low C:N ratio mung bean treatment (Table 1). Figure 2 is a conceptual model of *S. coelicolor* A3(2) metabolism after 24 h of incubation in response to the low C:N ratio mung bean residues, with metabolic intermediates and differentially-expressed proteins highlighted.

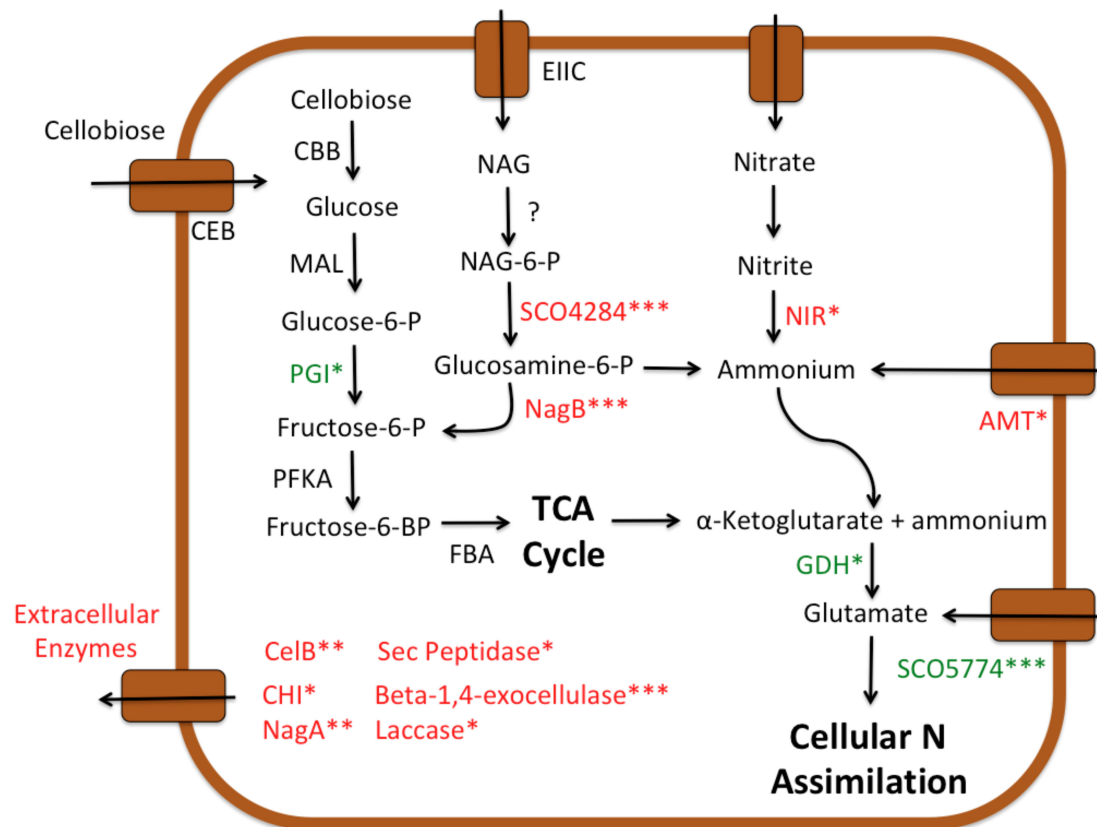


Figure 2. A conceptual model of changes in transcription in response to mung bean. Proteins labelled in green indicate significantly increased transcription, whereas red indicate significantly decreased transcription, for the comparison of wheat versus mung bean treatments with a Student’s *t*-test after 24 h of incubation. (***) $p \leq 0.001$; (**) $p = 0.001$; (*) $p \leq 0.05$. The question mark refers to an unknown enzyme in *S. coelicolor* A3(2).

Interestingly, the expression of a peptidase, *chiG*, and mineral N metabolism was reversed at day 7 and persisted up to day 14, with the mung bean treatment demonstrating increases relative to wheat (Table 1). At 14 days, the majority of extracellular enzymes were significantly expressed in the mung bean treatment. Also worthy of note was a switch in extracellular glutamate uptake from the permease *gluD* at 24 h to the ATP-dependent glutamate ATP-binding cassette (ABC) transporter *gluA* at days 7 and 14, which may indicate that the extracellular glutamate was no longer in excess compared to intracellular glutamate. The expression of *rpsB* remained relatively higher in the mung bean treatment over time, indicating higher general protein synthesis, however, it was not significant at days 7 and 14 (Table 1). These data indicate a delayed switch to N mining of SOM once labile, low C:N ratio plant material has likely been exhausted. While the mung bean treatment did demonstrate a delayed switch to nutrient mining, the exponentially decreasing rates of catabolism over time post substrate addition are a well-described phenomenon [11,38]; thus a delayed N mining effect seems preferable compared to an initial N mining effect in regard to retaining SOM.

The importance of N availability in regulating microbial metabolism has been explored thoroughly. The C:N ratio of the soil system, including plant material, is particularly important in determining both the formation and catabolism of SOM [5,10,39]. Initially, it was assumed that strict C:N stoichiometric controls would limit net decomposition i.e., N limitation would reduce heterotrophic activity, and conversely, N addition would increase net decomposition [40]. Numerous in situ studies demonstrated this to not necessarily be the case, suggesting that N addition inhibits the enzymes involved in lignin decomposition [41,42], resulting in the microbial community shifting away from the taxa directly involved in recalcitrant C breakdown [13,43], or shifting microbial investment in extracellular enzyme activity away from N acquisition (N mining) [8,9,44]. The increased expression of N-acquiring chitinase, N-acetyl glucosaminidase, and peptidases, as well as the increased expression of lignin-degrading laccase (a phenol oxidase) in the high C:N ratio wheat all agree with in situ studies that have demonstrated the increased activity of these enzymes under N limitation [44–48]. Interestingly, these studies also reported a general increase in C-acquiring cellobiohydrolase and glucosidase activity upon N addition, which did not occur here, as *S. coelicolor* A3(2) cellulase and cellobiohydrolase expression were also greater in the wheat at 24 h incubation. Given the simplified nature of this in vitro system that focused on the DGE of a model organism, it is possible that the response of these enzymes to N may be driven by other organisms within microbial communities. Thus, the relative importance of shifts in the composition of the microbial community in response to N addition may be enzyme-specific.

A potential means to reduce SOM losses from agricultural soils involves the reduction of the C:N ratio of the system, either through appropriate mineral and/or organic fertilisation [49–51] or crop rotations with N₂-fixing plant species [52,53]. The results demonstrated here suggest a particularly important role for organic N in the form of glutamate. However, it should be noted that other important factors affect SOM turnover, including the molecular composition of the input plant material and both environmental and edaphic factors that regulate SOM accessibility [6,54,55].

4. Conclusions

Here we demonstrate a suite of transcribed genes that are supportive of the N mining hypothesis. In short, under relatively N-limiting conditions, *S. coelicolor* A3(2) increased the transcription of genes involved in C and both organic and mineral N acquisition after 24 h. Despite this, *S. coelicolor* A3(2) assimilated less cellular N through GDH and transcribed less transporters for glutamate uptake relative to non N-limiting conditions. After 14 days, N mining had increased in the low C:N ratio treatment as a delayed response to N requirements. This emphasises the importance of regulating the C:N stoichiometry of the soil system in order to improve the management of SOM.

Author Contributions: Author contributions are as follows: conceptualization, D.F., P.M.K. and R.C.D.; methodology, D.F.; software, D.F.; validation, D.F. and K.C.; formal analysis, D.F. and M.H.; investigation, D.F.; resources, D.O., A.V.K. and R.C.D.; data curation, D.F.; writing—original draft preparation, D.F.; writing—review and editing, P.M.K., A.V.K. and R.C.D.; visualization, D.F.; supervision, R.C.D.; project administration, R.C.D.; funding acquisition, R.C.D.

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Conflicts of Interest: The authors declare no conflicts of interest.

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