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Rhizosphere and detritusphere habitats modulate expression of soil N-cycling genes during plant development

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ABSTRACT Interactions between plant roots and rhizosphere bacteria modulate nitrogen (N)-cycling processes and create habitats rich in low molecular weight compounds (exudates) and complex organic molecules (decaying root litter) compared to those of bulk soil. Microbial N-cycling is regulated by edaphic conditions and genes from many interconnected metabolic pathways, but most studies of soil N-cycling gene expression have focused on single pathways. Currently, we lack a comprehensive understanding of the interplay between soil N-cycling gene regulation, spatial habitat, and time. We present results from a replicated time series of soil metatranscriptomes; we followed gene expression of multiple N transformations in four soil habitats (rhizosphere, detritusphere, rhizo-detritusphere, and bulk soil) during active root growth for the annual grass, *Avena fatua*. The presence of root litter and living roots significantly altered the trajectories of N-cycling gene expression. Upregulation of assimilatory nitrate reduction in the rhizosphere suggests that rhizosphere bacteria were actively competing with roots for nitrate. Simultaneously, ammonium assimilatory pathways were upregulated in both rhizosphere and detritusphere soil, which could have limited N availability to plants. The detritusphere supported dissimilatory processes DNRA and denitrification. Expression of nitrification genes was dominated by three phylotypes of *Thaumarchaeota* and was upregulated in bulk soil. Unidirectional ammonium assimilation and its regulatory genes (GS/GOGAT) were upregulated near relatively young roots and highly decayed root litter, suggesting N may have been limiting in these habitats (GS/GOGAT is typically activated under N limitation). Our comprehensive analysis indicates that differences in carbon and inorganic N availability control contemporaneous transcription of N-cycling pathways in soil habitats.

IMPORTANCE Plant roots modulate microbial nitrogen (N) cycling by regulating the supply of root-derived carbon and nitrogen uptake. These differences in resource availability cause distinct micro-habitats to develop: soil near living roots, decaying roots, near both, or outside the direct influence of roots. While many environmental factors and genes control the microbial processes involved in the nitrogen cycle, most research has focused on single genes and pathways, neglecting the interactive effects these pathways have on each other. The processes controlled by these pathways determine consumption and production of N by soil microorganisms. We followed the expression of N-cycling genes in four soil microhabitats over a period of active root growth for an annual grass. We found that the presence of root litter and living roots significantly altered gene expression involved in multiple nitrogen pathways, as well as tradeoffs between pathways, which ultimately regulate N availability to plants.

KEYWORDS soil microbiome, soil nitrogen, rhizosphere, gene expression, metatranscriptomics, nitrification, plant litter, detritusphere

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Nitrogen (N) is a key limiting nutrient for plant growth, since in soil, N mostly occurs in organic forms as large, complex molecules that are unavailable to plants. Soil microorganisms have suites of extracellular enzymes capable of degrading these molecules, yielding small organic and inorganic N to the surrounding soil, and benefitting nearby plants. Some bacteria and archaea oxidize N to nitrate—an inorganic form that, like ammonium, is available to many plants. Meanwhile, microorganisms also take up inorganic N for a variety of growth-supporting assimilatory and dissimilatory metabolic processes. This can lead to some competition for N between soil bacteria and plants as well as between different soil bacteria. While many of these processes occur simultaneously in soil, we have an incomplete understanding of how they vary with time and in distinct soil habitats.

Soils contain numerous microhabitats with heterogeneous distributions of substrates and resources (1). The influence of growing roots can dominate (rhizosphere), or dead root litter may dominate (detritusphere); alternatively, these two habitats may co-occur when live roots regrow into previously colonized areas (rhizo/detritusphere). In previous analyses, we have shown that distinct bacterial guilds operate in rhizosphere, detritusphere, and combined rhizosphere-detritusphere soil habitats (2, 3). The spatial organization of these soil habitats may be particularly important for nutrient transfers that create distinct microhabitats that enable or limit N-cycling. For example: (i) in the rhizosphere, exudate-driven blooms of microbial growth are followed by predation that liberates the nutrient capital held in bacterial, fungal, and metazoan bodies; this redistribution of microbial biomass causes conversion of lower molecular weight compounds (e.g., amino acids) that are deaminated to ammonium by diverse bacteria and fungi (4, 5); (ii) in the detritusphere, organic N in root litter [e.g., lignoproteins (6)] is mineralized to make inorganic N available to soil microbes, while the same organic material serves as a carbon (C) source for soil microbes (7), creating a demand for N to maintain cellular stoichiometry; and (iii) in bulk soils, some microbes may express more macromolecule degradation genes relative to more resource-rich sites near roots, where degradation enzymes for low molecular weight compounds are more prevalent (8).

Rates of microbial N transformations in soil can be significantly impacted by soil edaphic conditions (e.g., moisture, pH, O_2, and texture) and the presence of both growing and decaying roots. Gross N mineralization, immobilization, and nitrification rates vary as a function of proximity to plant roots, root age, and concentration of organic material in grassland soil (9, 10). Roots can significantly deplete ammonium in the rhizosphere within days of root introduction into sterile soil (11). However, in wild soils with intact microbial communities, rhizosphere-associated bacteria can transiently compete with plants (e.g., *Avena barbata* roots) for inorganic nitrogen (12). As part of this competition, plants can inhibit rhizosphere nitrogen transformations through mechanisms such as biological nitrification inhibition (BNI) by production of certain root exudate compounds (13). Plants have also been shown to inhibit both nitrification and denitrification processes in the rhizosphere—a combination of processes that leads to loss of N from the plant-available pool (14)—and litter amendment has been shown to increase denitrification (15). Understanding the N-cycling tradeoffs between different soil habitats is key for our ability to better model and predict the controls of soil N-cycling.

Soil metatranscriptome analysis is a powerful tool for linking nitrogen cycling genes to specific microbial populations and correlating transcription levels to environmental conditions (2, 3, 16–19). In comparison, qPCR can target only single genes, and primer biases may leave some taxa undetected. A recent metatranscriptomics study demonstrated a short-term coupling of nitrogen cycling pathways and availability of simple carbons simulating priming by root exudates (20). Otherwise, there is little knowledge of the contemporaneous feedback through gene expression between carbon availability and nitrogen cycling in naturally complex soil habitats and over annual plant-relevant time frames (21–23).

Here, we used soil metatranscriptomics analysis to build a comprehensive time-resolved representation of both organic and inorganic nitrogen transformations in an annual grassland soil. We characterized a period of active root growth, as well as root decomposition, of wild oat grass (*Avena fatua*), a common species in Mediterranean grasslands. We hypothesized that different N cycle pathways would be prevalent in different soil microhabitats that vary in available C and N resources. We analyzed 48 metatranscriptomes from rhizosphere and bulk soil collected over a 3-week time series in the presence or absence of root litter; this allowed us to identify the dominant N-cycling pathways associated with each habitat and untangle the effects of carbon supply by live or dead roots from competition with the plant for N. We previously analyzed a different subset of these data to assess expression of genes coding for carbohydrate degradation (2) and complex organic nitrogen degrading enzymes (3). While these prior studies provide important context, our analysis here is unique in its comprehensive exploration of how N transformation gene expression is influenced by the distinct environmental conditions of different soil habitats. The use of metatranscriptomics (as opposed to qPCR) also facilitated identification of specific microbial populations involved in each pathway and helped us test for multiple N transforming pathways expressed within populations found in multiple soil habitats. Finally, we measured changes in N pathway gene expression over time as the rhizosphere aged and as root litter was degraded.

MATERIALS AND METHODS

Experimental design, sample collection, and sequencing

The experimental procedures and initial data processing for our 48 soil metatranscriptome data set are described in detail in reference (2). Briefly, *Avena fatua* was grown in a fine loam Alfisol complex (Ultic Haploxeralf mixed with a Mollic Palexeralf) from the Hopland Research and Extension Center (Hopland, California), pH 5.6, 2% total C, in microcosms with a sidecar with transparent walls. After 6 weeks, roughly halfway through the plant life span, the solid divider to the sidecar was removed and replaced with a slotted divider so that roots could grow into the sidecar. Root growth was marked on the sidecar wall. All sidecars contained bulk soil bags that were inaccessible to roots. Half of the bulk soil bags as well as the soil in half of the sidecars were amended with dried *A. fatua* root detritus (litter C:N = 13.4). Once the sidecar was opened, paired rhizosphere and bulk soil triplicates were harvested destructively after 3, 6, 12, and 22 days from amended and unamended microcosms for a total of 48 samples. Lifeguard Soil Preservation Reagent (MoBio) was added to a 1 g subsample of the harvested soil. The supernatant and roots were removed, and the samples were stored at −80°C.

DNA/RNA co-extraction was performed with phenol-chloroform. DNA and RNA were separated with an AllPrep Kit (Qiagen), and RNA was DNase treated (TURBO DNase, Thermo Fisher Scientific). Ribosomal RNA was depleted (RiboZero, Illumina), and the mRNA was retrotranscribed. cDNA was sequenced with an Illumina HiSeq 2000 2 × 150 (TruSeq SBS v3) protocol at the Joint Genome Institute. In addition, the V4 hypervariable region of the 16S gene (primers 515F and 806R) was amplified from the extracted DNA and sequenced via Illumina MiSeq v3 2 × 300. Amplicons were analyzed by the Joint Genome Institute. Operational taxonomic units (OTUs) were clustered at 97% identity with USEARCH (24), and taxonomy was assigned at 95% ID by RDP (25).

Expression of nitrogen cycling genes identified in assembled metatranscriptomes

After quality control, the metatranscriptomes were assembled into contigs within each sample. Only contigs larger than 200 bp were clustered at 99% ID with cd-hit-est (26). Prodigal (26, 27) was used to predict open reading frames (ORFs) from the cluster representatives (longest contig), and KEGG models (28) were used to locate nitrogen cycling genes based on the KEGG nitrogen cycle module (see Table S1 for a list of

KEGG profiles, gene lengths, and thresholds used). Some ORFs were identified by several HMMs due to homology (e.g., nitrate reductases *nxrA*, *narG*, and *napA*). In those cases, the highest-scoring (by bit score) hit from all HMMs was selected. To further verify the annotations, we placed the assembled sequences on a protein reference tree (29) and corrected the annotations according to tree clustering. Notably, assignment by the highest bit score was generally correct. Extracellular proteases were called by reciprocal blast to proteases from the MEROPS database that contained signal peptides (30, 31). Reads were mapped to nitrogen cycling ORFs at a minimum identity of 95% and minimum breadth of 75% using bbmap (2, 32). Normalization to sequencing depth and gene length was done with DESeq2. Heat maps were generated in R with gplots (33).

Phylogeny of the ammonium monooxygenase subunit A (*amoA*) was determined by aligning *amoA*-assembled transcripts to curated full *amoA* gene sequences from RefSeq (Jun 1, 2019) using mafft-linsi (sensitive mode) (34) and removing positions represented by less than 50% of the aligned sequences with GBlocks version 0.91 (35) with parameters -b3=50 -b4=5 -b5=h. RAxML version 8.2.12 (36) with parameters set to matrix LG and seed = 13 was used to build a Newick tree which was visualized in iTOL (37). The phylogeny of *nirK* transcripts was determined in the same manner. The aggregated coverage of *amoA*/*nirK* variants from all samples was superimposed onto the tree in iTOL. As the bacterial *nirK* participates in denitrification whereas its archaeal homolog functions in nitrification, we separated *nirK* variants and their expression by the pathway they participate in and tested amendment, location, and time effects by pathway. Tables were constructed and manipulated with tidyverse (38), and figures were generated with ggplot2 (39). Variance-normalized expression per gene per timepoint was compared between groups using ANOVA and Tukey HSD tests, and *P*-values were FDR adjusted for multiple comparisons (number of genes, time, location, litter amendment, and all interactions between them). Upregulation was determined as log2-fold values calculated by DESeq2 compared to unamended bulk soil at the same timepoint.

All features and their metatranscriptomic read recruitment were analyzed with the R package DESeq2 (40, 41) requiring an adjusted $P < 0.05$. Ordination and visualization were conducted using R package ggplot2 (39) and vegan (39, 42); the vegan function Adonis was used to perform PERMANOVA on DESeq2 counts (variance normalized to the gene length and sequencing depth) to detect significant factors affecting expression of nitrogen cycling genes.

RESULTS

To assess whether expression of organic and inorganic nitrogen cycling genes was affected by the presence of root litter in the rhizosphere or in nearby bulk soil, we tested for significant differences in N transformation gene expression over a 3-week time series (3, 6, 12, and 22 days). Unlike prior analyses of this metatranscriptome data set, where we focused on carbohydrate-active enzymes (CAZy) (2) or proteases and chitinases (3) and mapped transcriptomic short reads to a collection of curated genomes, in our current study, we focused on assembled transcripts of all nitrogen cycling genes, in order to capture more of the genetic diversity present in the system. A principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity of expression of 57,469 N-cycling gene variants normalized to sequencing depth revealed that the last timepoint (T4; 22 days) was notably distinct from the others (Fig. 1A). Analysis of the first three timepoints only (3, 6, and 12 days) revealed additional clustering by litter amendment and location (rhizosphere/bulk soil) (Fig. 1B). In both analyses, the first two principal components explained 40% of the variation. PERMANOVA analysis using transcript counts variance normalized to the gene length and sequencing depth from all timepoints indicated a significant effect of location (rhizosphere vs bulk soil), time, and litter amendment ($P = 10^{-4}$) (see Table S1 for all values). The combined R^2 of all significant factors was 63%. When we conducted the same analysis but excluded the last timepoint (T4), the effects of single factors remained significant ($P = 10^{-4}$). The combined R^2 of all significant factors was 61%, and the variability explained by time decreased from 30% with T4 to 11%

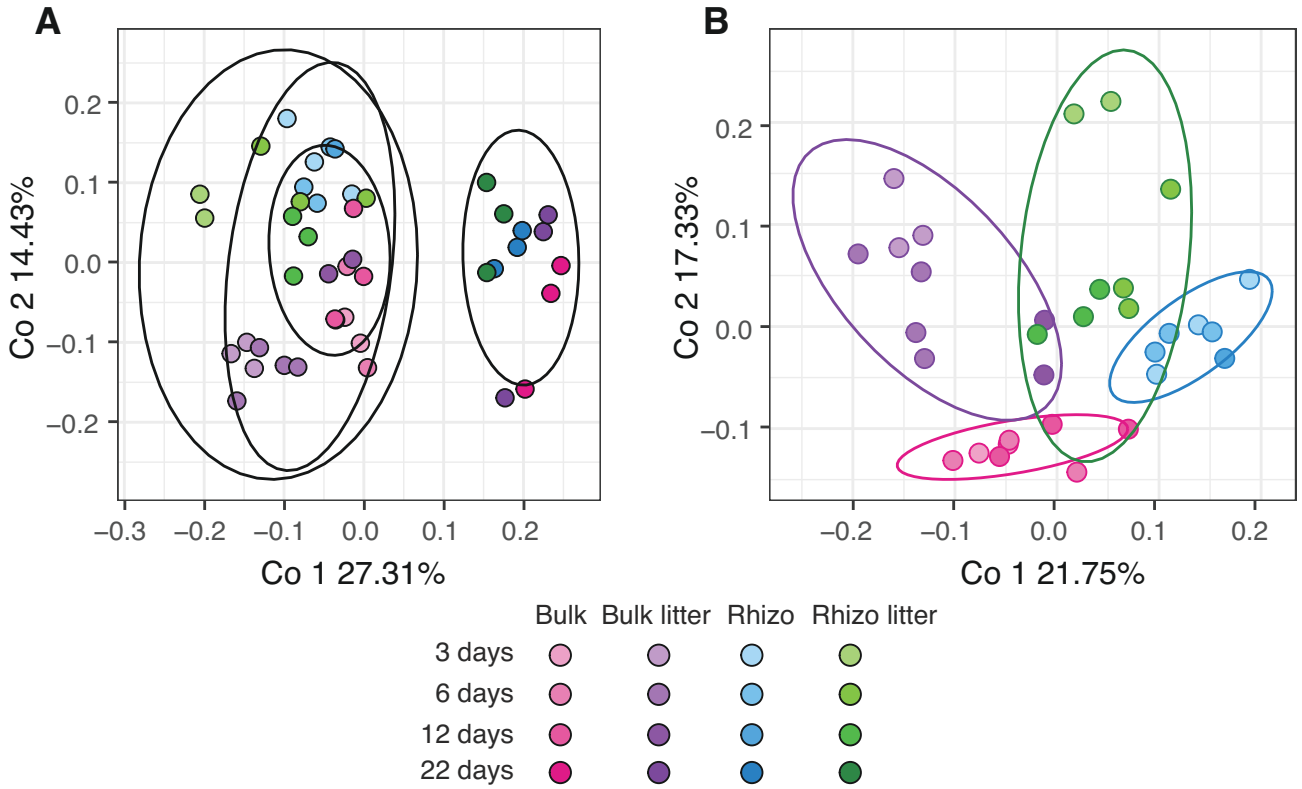

FIG 1 Principal coordinates analysis of nitrogen cycling gene expression. Influence of living roots and root detritus on soil microbial gene expression during 3 weeks of *Avena fatua* root growth (harvests at 3, 6, 12, and 22 days) in a California annual grassland soil. Input data are Bray-Curtis dissimilarity of expression of N-cycling genes variance normalized to the sequencing depth and gene length, determined by mapping reads to ORFs assembled from metatranscriptomes, in (A) all timepoints and (B) excluding the last timepoint (22 days). n = 3 for each habitat and timepoint. Ellipses represent a 90% confidence interval of the ordination coordinates (calculated by stat_ellipse).

without T4, whereas the variability explained by location and amendment increased by 8% and 6%, respectively (Table S1).

Expression of nitrogen cycling genes by pathway

To identify the dominant N-cycling pathways in four soil microhabitats (rhizosphere, detritusphere, rhizo/detritusphere, and bulk soil), we calculated the $\log_2$ ratio differential expression of each nitrogen cycling gene in the presence or absence of roots and litter amendment to expression in unamended bulk soil at the same timepoint. Expression and upregulation trends of N-cycling genes (defined in Table S2, comparison by ANOVA and Tukey post-hoc tests with *P* values FDR adjusted for multiple comparisons, Table S3) are shown in Fig. 2. Several genes involved in assimilation of inorganic N were significantly upregulated in the rhizosphere compared to bulk soil: assimilatory nitrate reduction genes *nasA*, *NR* and *nirA*, glutamate dehydrogenase *gdh2*, and GS/GOGAT genes *glt1* and *gltB* (Fig. 2). The first step of GS/GOGAT, *glnA*, was upregulated both in the rhizosphere and in the presence of litter compared to unamended bulk soil (Fig. 2), and the GS/GOGAT regulatory proteins *glnD* and *glnG*, triggered under N limitation, were upregulated in the rhizosphere, whereas GS/GOGAT activator *glnB* was upregulated in the presence of litter (Fig. S1). In the detritusphere, the presence of litter also triggered macromolecular organic nitrogen degradation (extracellular protease and chitinase), as well as dissimilatory pathways, dissimilatory nitrate reduction (DNRA) and denitrification compared to unamended bulk soil (Fig. 2). However, not all genes in these pathways were upregulated, and some were not detected at all (*nirS* and *norBC* in denitrification), whereas others were not significantly upregulated (*napB*) (Fig. 2; Table S3). Finally,

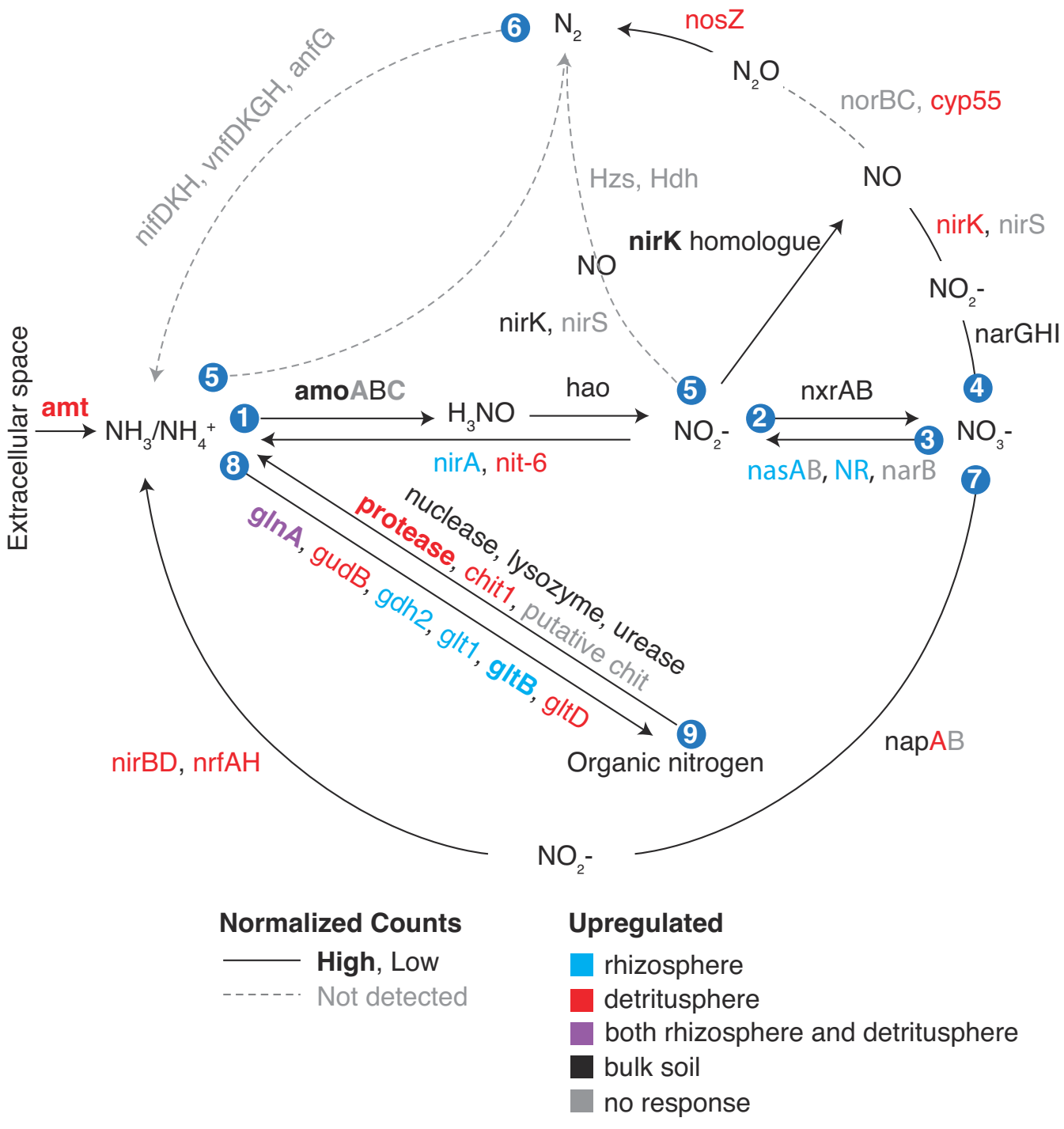

FIG 2 Expression level and upregulation by soil habitat of nitrogen cycling genes from assembled transcripts. Bold font indicates high expression levels (mean counts in each timepoint and habitat >5 × 10^4 after normalization to the gene length and sequencing depth). Significant upregulation compared to unamended bulk soil at the same timepoint was determined by ANOVA and Tukey post hoc test (adjusted $P < 0.05$; Table S3). Numbers denote pathways: 1, ammonium oxidation; 2, nitrite oxidation; 3, assimilatory nitrate reduction; 4, denitrification; 5, anammox; 6, nitrogen fixation; 7, DNRA; 8, ammonium assimilation (GDH and GS/GOGAT); 9, macromolecular N mineralization; amt, ammonium transporter. The functional role of each enzyme is explained in Table S2. The *nirK* homologue is an archaeal enzyme which participates in archaeal nitrification, but unlike its bacterial homologue, it does not generate nitrate (43).

nitrification, DNRA, and the first step of denitrification were upregulated in bulk soil compared to the rhizosphere (Fig. S1; Table S3).

Several genes were particularly highly expressed in our data set [e.g., ammonium monooxygenase subunits *amoA* and *amoC*, and the archaeal homologue of nitrate reductase *nirK* (Fig. S1)] but did not show clear patterns of differential expression by either amendment or time. We did not detect any genes involved in nitrogen fixation or anaerobic ammonium oxidation. Expression of fungal N cycle genes such as *cyp55* (nitric oxide to nitrous oxide) and *nit-6* (nitrite to ammonium) was very low (Fig. S1).

Archaeal nitrification

Two of the most highly expressed genes we identified were subunits of ammonium monooxygenase, the first enzyme in the nitrification pathway. Nitrification genes were not affected by the presence of litter, but *nirK*, *amoB*, *hao*, *nxrA*, and *nxrB* were significantly upregulated in bulk soil compared to rhizosphere soil (ANOVA: $P < 0.05$; Tukey HSD test: adjusted $P < 0.05$) (Table S2).

Ammonium oxidation can be performed either by ammonium-oxidizing archaea (AOA) or ammonium-oxidizing bacteria (AOB). The *amoA* subunit of ammonium monooxygenase is commonly used as a taxonomic marker to differentiate between AOA and AOB. To determine which taxa were responsible for the high expression of ammonium oxidation genes, we placed the 13 *amoA* ORFs identified in assembled transcripts into a phylogenetic tree with full-length *amoA* reference sequences from the RefSeq database (Fig. 3A). The cumulative coverage per *amoA* variant in all samples was superimposed onto the tree. Read recruitment to AOA was orders of magnitude higher than to AOB, accounting for 98% ± 0.007% of the reads mapped to *amoA* per sample ($n = 16$). While there were some ORFs placed into comammox clade B, their expression was negligible compared to that of archaeal *amoA* (Fig. 3A).

The expression of hydroxylamine oxidoreductase (*hao*), the next step in bacterial nitrification after ammonium oxidation, was extremely low across all samples. Similarly, expression of nitrite oxidase (*nxrAB*) was very low. However, the expression of *nirK* was very high (Fig. 2). A phylogenetic analysis placed 96 out of 158 *nirK*-assembled ORFs in the archaeal clade and, as in the case of *amoA*, revealed that the archaeal variants recruited more than 95% ± 0.07% of the reads that mapped to *nirK* (Fig. 3B) ($n = 16$). Three archaeal phylotypes dominated *nirK* expression, with 76% ± 0.07% of all reads mapped to *nirK* ($n = 16$).

To determine whether the high expression of archaeal nitrification genes can be explained by the abundance of AOA compared to AOB, we searched our *16S rRNA* gene amplicons (2) for known AOA and AOB. The relative abundance of AOA *16S rRNA* genes was two- to fivefold higher than that of AOB, and the relative abundance of AOB was consistently higher than that of nitrite-oxidizing bacteria (NOB) (Fig. 3C). The aggregate expression of *amoA* and *nirK* was well correlated with a ratio of *nirK*:*amoA* = 1.6 ($R^2 = 0.7$, $P < 0.0001$) (Fig. 3D). In addition, the aggregated expression of *amoA* was also highly correlated to that of Thaumarchaeal extracellular proteases (3) (Fig. 3E; adjusted $R^2 = 0.61$, $P < 0.0001$). Finally, the expression of *amoA* in the unamended rhizosphere decreased near aging roots (22 days; Fig. 3F).

Ammonium assimilation and transport pathways

The most highly expressed ammonium acquisition pathway was glutamate synthase (GS/GOGAT), which can be activated by a cascade of four enzymes under nitrogen limitation (Fig. 4A) (44–46). As opposed to single-gene-dependent transporters (*amt*) and glutamate dehydrogenase (GDH), GS/GOGAT is a two-step pathway: the first step is encoded by the gene *glnA* and the second step by either *glt1*, *Glu*, or the heterodimer *gltBD*. Both ammonium transporters (Fig. S2) and GS/GOGAT (*glnA*; Fig. 4B) were significantly upregulated in the young litter-amended rhizosphere (Rhizo Litter, 3 days) and in bulk soil at the final timepoint (Bulk Litter, 22 days) (Fig. 4B). *glnA* was also significantly upregulated in the early rhizosphere compared towith the bulk control (Rhizo; Fig. 4B), and three out of four detected proteins involved in GS/GOGAT were also upregulated in the rhizosphere after 3, 6, and 12 days (Fig. S3). We found that expression of *gltBD* was

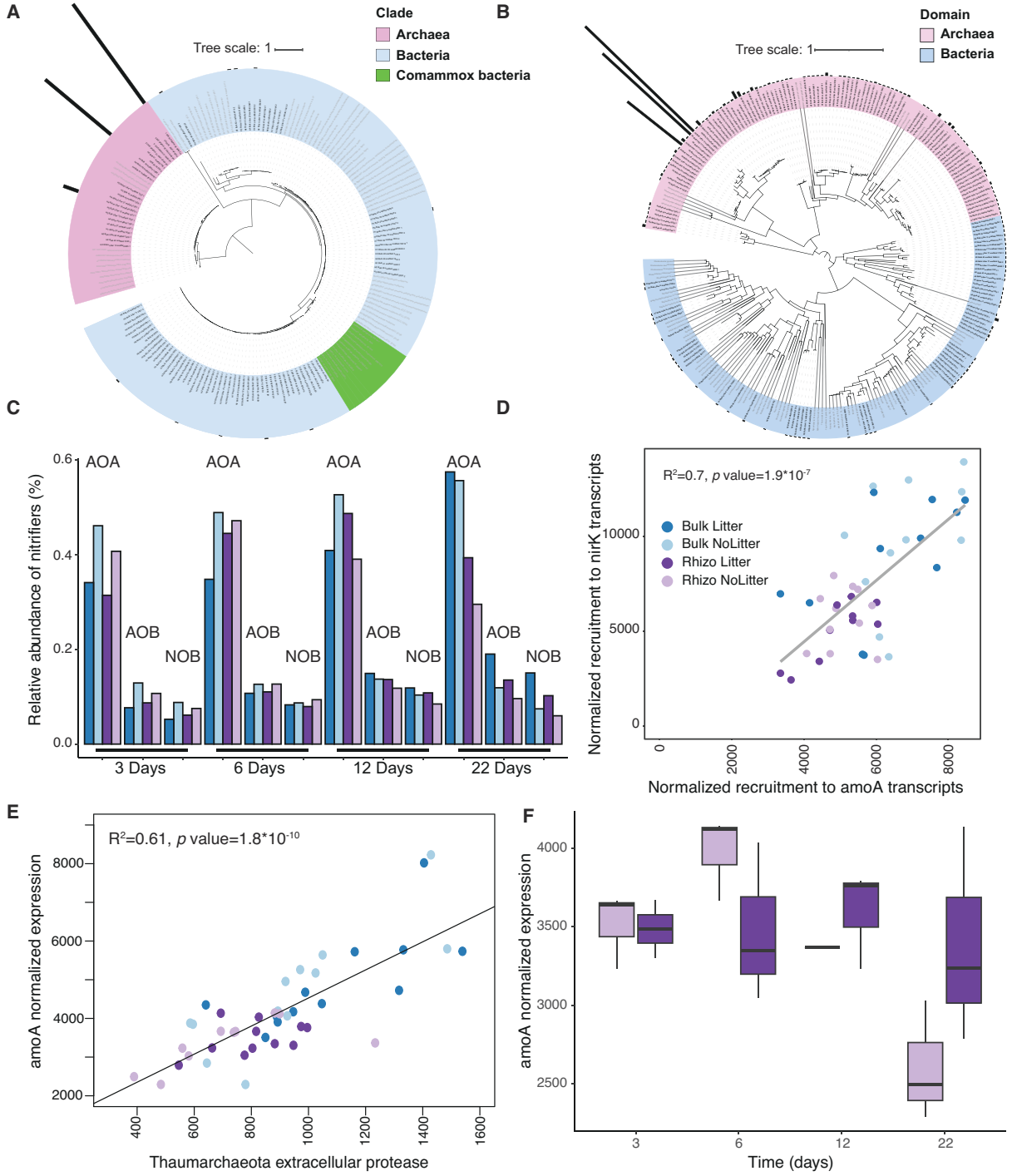

FIG 3 Phylogenetic determination of nitrifiers by *amoA*, *nirK*, and *16S rRNA*. Phylogeny of assembled ORFs (black) (A) *amoA* and (B) *nirK* placed into phylogenetic trees containing curated reference sequences from RefSeq (gray). Colors represent phylogenetic clades. Bars around the trees represent cumulative coverage from all samples to specific assembled transcripts. (C) Relative abundance of AOA, AOB, and nitrite oxidizing bacteria (NOB) of the entire microbial community by *16S-rRNA* gene abundance. (D) Positive correlation between expression of *amoA* and *nirK* transcripts by location and amendment (R^2 = 0.7). (E) Positive correlation between expression of *amoA* and *Thaumarchaeota* extracellular protease transcripts by location and amendment (R^2 = 0.61). (F) Normalized expression of *amoA* in the rhizosphere (with or without litter amendment) over time. The legend in panel D applies to panels C–F.

generally much higher than that of *glt1*, whereas *Glu* was not detected (Fig. S1). Expression of an additional ammonium assimilation pathway, glutamate dehydrogenase, was low (Fig. S1). GDH consists of a single step performed by one of two enzymes: *gdh2* or

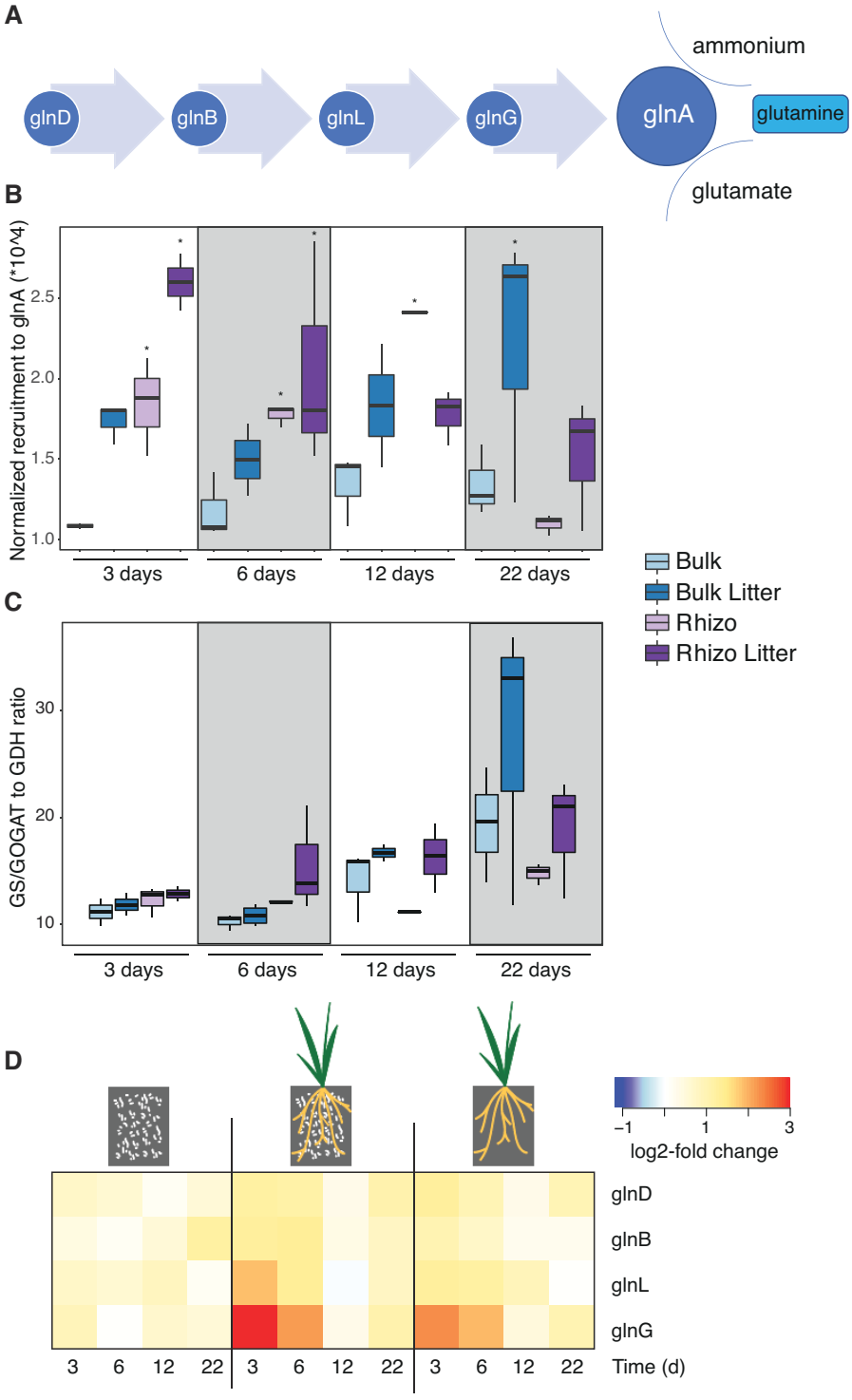

FIG 4 Expression of ammonium assimilation pathways and their regulatory genes. (A) Simplified conceptual representation of *glnA* activation via the regulatory cascade. (B) Variance-normalized expression of *glnA* in amended/unamended rhizosphere and bulk soil. Asterisks denote a significant increase compared to the same litter amendment in bulk soil (e.g., unamended rhizosphere vs unamended bulk). (C) Ratio of GS/GOGAT to GDH (*glnA:gudB*) expression in the metatranscriptomes. (D) Mean log ratio transformed differential expression compared to unamended bulk soil of *glnA,* and the regulatory genes that activate it under N limitation.

gudB. *Gdh2* was upregulated in the rhizosphere, whereas *gudB* was upregulated in the presence of litter (Fig. S1; Table S2).

Theoretically, there is an advantage to using GS/GOGAT for ammonium assimilation over GDH under N limitation, as GS/GOGAT in a one-way pathway, whereas GDH can also catalyze deamination of glutamate which might lead to loss of ammonium. Expression of GS/GOGAT was an order of magnitude higher than that of GDH under all experimental conditions (Fig. 4C). The ratio of expression (GS/GOGAT to GDH) significantly increased over time [PERMANOVA model: ratio~condition×time, P(time) = 0.002]. In the young rhizosphere, the ratio appeared to be higher compared to that in bulk soil, whereas in the mature rhizosphere, litter seemed to trigger a higher ratio (Fig. 4C). Expression of the GS/GOGAT regulatory proteins *glnD*, *glnG*, and *glnL* was significantly upregulated in the rhizosphere compared to bulk soil, particularly in the young rhizosphere (Fig. 4D; Table S3). Most regulatory genes were also upregulated in the detritusphere compared to unamended soil at the last timepoint (Fig. 4D).

DISCUSSION

In this study, we used metatranscriptomic gene expression (percentage of reads mapped to a gene) and upregulation (log-fold change of expression in a habitat compared to unamended bulk soil) to identify contemporaneous N transformations in four soil habitats: rhizosphere (near live roots), detritusphere (near dead roots), rhizo-detritusphere (intermingled live and dead roots), and bulk soil (without root influence). The expression of genes coding for N-cycling processes varied both between soil habitats and over a relatively short time frame. The use of metatranscriptomic analyses allowed us to track multiple N-cycling pathways occurring at the same time. Moreover, as N pools in this grassland soil are relatively constant but fluxes are high (47), studying N-cycling gene expression may be a better proxy for flux rates than pools.

Using microbial gene expression to monitor how roots and root litter affect N-cycling allowed us to explore N pathway activity at a spatial and temporal resolution that would be difficult for more traditional approaches such as enzyme activity assays or isotope tracing approaches which require volumes of soil that are difficult to collect in a microhabitat such as the rhizosphere. Metatranscriptomics also helps to point out "realized" metabolic niches for individual taxonomic groups within a complex microbial community (2). Until recently, niche differentiation has been difficult to describe for soil microorganisms due to their high degree of functional redundancy and the fact that most soil microbes cannot be cultured in the laboratory. While metatranscriptomics remains a relatively new approach for soil nitrogen cycling studies (20, 48, 49) and has rarely been benchmarked against more established methods [e.g., enzyme assays, although see reference (50)], it can help to target the more active members of a community, allows simultaneous detection of activity across all domains of life (51), and alleviates many of the concerns associated with relic DNA (52). As we have previously shown with this data set, transcriptional succession may occur at a faster pace than taxonomic succession (2). Thus, transcript analyses may be a particularly sensitive readout of community response to current edaphic conditions and resource availability.

Our results illustrate how soil microhabitat and temporal patterns of N-cycling gene expression are tightly interrelated. We detected the strong effects of habitat on gene expression at the first three sampling times, although not after 22 days when the soil had become drier due to plant transpiration (2). This decrease in soil moisture likely affected rates of substrate diffusion as well as microbial mobility, leading to changes in the edaphic conditions experienced by the microbes. In addition to reduced soil moisture, the roots present in the rhizosphere have begun to senescence after 22 days and thus reflect degradation processes that are more characteristic of a root litter habitat. Moreover, root exudation decreases as a root senesces, and the shift from microbial exploitation of simple exudate carbon sources to usage of root litter may be another cause of the temporal shifts we observed (8). After removing the last timepoint from analyses, we observed strong effects of roots and litter on gene expression, as well as

the interaction between the two, implying that rhizosphere bacteria responded to litter amendment differently than bulk soil bacteria. These results suggest that N process rates are not driven solely by the soil habitat, or by plant development, but that the two are interrelated, and gross rates of N-cycling processes are likely changing faster in space and time than previously thought.

Several N-cycling pathway genes were differentially expressed in different soil habitats. In the rhizosphere, we identified upregulation of assimilatory nitrate reduction and ammonium assimilation through glutamate dehydrogenase (GDH; *gdh2*) and glutamine synthetase (GS/GOGAT; *glnA*, *glt1*, and *gltB*). An additional GDH enzyme, *gudB*, was upregulated in the litter-amended rhizosphere. Three of four regulatory enzymes that activate GS/GOGAT under N limitation were also upregulated in the rhizosphere, and the fourth was upregulated in the litter-amended rhizosphere. The upregulation of these pathways likely indicates that the rhizosphere microbial community was experiencing inorganic N limitation. N limitation can stem from competition over inorganic N with the plant (12, 53), as well as from a higher demand for N as microbial density is ca. 10× higher in the rhizosphere compared to bulk soil (54). *Avena* plants have been shown to be better competitors than their rhizosphere community for nitrate (12, 53), potentially explaining why rhizosphere microorganisms would upregulate assimilatory nitrate reduction to ammonium.

Compared to bulk soil, dissimilatory nitrate reduction (DNRA) and denitrification pathways were upregulated in the detritusphere. Denitrification leads to loss of gaseous nitrogen from the system either as N_2, N_2O, or NO, whereas DNRA is a N-conservative process, converting NO_3 (that can be easily lost) into less-mobile ammonium. A recent meta-analysis suggests DNRA is highly sensitive to carbon substrate supply and precipitation (a proxy for oxygen availability) and negatively related to nitrous oxide (N_2O) emissions (55), implying that it is negatively correlated to denitrification. However, we find that both denitrification and DNRA were promoted in the detritusphere.

As N is commonly a limiting nutrient in the rhizosphere, coveted by both plants and microbes, denitrification and the resulting loss of N might be disadvantageous to plants and N-limited microorganisms. Indeed, our data indicate downregulation of denitrification in the rhizosphere compared to bulk soil. Plants have been demonstrated to outcompete nitrate-reducing bacteria for nitrate (56), and there is some evidence that root exudates can inhibit denitrification (57). However, multiple studies have hypothesized that denitrification activities may be stimulated by the addition of root exudates (58) and even predict that exudate composition shapes the gaseous end-product of denitrification and thus greenhouse gas emissions (59). Our data show that some of the genes involved in denitrification and DNRA were also significantly upregulated in litter-amended bulk soil compared to litter-amended rhizosphere, also suggesting the inhibition of dissimilatory processes in rhizosphere soil.

Upregulation of assimilatory processes (nitrate reduction and ammonium assimilation) in the detritusphere compared to unamended soil was likely driven by the use of carbon substrates to maintain C:N cellular stoichiometry. Unlike in the rhizosphere, where there is competition between microbes and plants for nitrate, in the detritusphere, the driving force should be only carbon and oxygen consumption which may create anaerobic or microaerobic niches that would support DNRA and denitrification. Expression of assimilatory process genes that were upregulated in the detritusphere compared to bulk soil (*gudB*, *gltD*) was higher than that of any of the DNRA or denitrification genes, suggesting that dissimilatory nitrate reducers may be outcompeted by assimilatory nitrate reducers in the detritusphere, likely due to oxygen and carbon availability.

In bulk soils, genes coding for nitrification were upregulated, leading to potential loss of N from the system via generation of nitrous oxide and nitrate leaching (60). Ammonium monooxygenase subunit C (*amoC*) was one of the most highly expressed genes in this soil. Interestingly, the expression of subsequent steps in bacterial nitrification (*hao*, *nxrA*, and *nxrB*) was extremely low. However, expression of *nirK*, traditionally considered

a part of the denitrification process, was one of the highest we detected. When we explored the phylogeny of detected *amoA* and *nirK* variants, we discovered that all highly transcribed variants were archaeal. *nirK* has been detected in archaeal genomes, and the current leading hypothesis is that archaea oxidize hydroxylamine by combining it with nitric oxide (NO), yielding two nitrite molecules, one of which is reduced and recycled back to NO by *nirK* (60). While it is possible that conditions are more favorable for nitrification in bulk soil because there is less competition for ammonium in the absence of a plant, it is also possible that nitrification was downregulated or inhibited in the rhizosphere. Biological nitrification inhibition (BNI) by the exudation of secondary metabolites from plant roots can suppress ammonium oxidation by nitrifying microorganisms (61, 62), thus increasing the availability of ammonium to plants. Hence, it is proposed as one strategy to increase crop yields and reduce fertilizer loss (63). Root exudates of *A. fatua* have been shown to inhibit nitrification by ammonium-oxidizing bacterium *Nitrosomonas europaea* (64). If *A. fatua* inhibited ammonium-oxidizing archaea to increase ammonium availability to its roots, this would likely have presented as upregulation in bulk soil. Our observations of N transcript expression in the rhizosphere and bulk soil suggest that microbes in the rhizosphere experience N limitation, as well as competition over ammonium with the plant, thereby minimizing nitrification.

Despite the low relative abundance of AOA, archaeal genes for ammonium monooxygenase (*amoABC*) were among the most highly expressed of all N-cycling transcripts in this study across time, location (bulk soil vs rhizosphere), and presence or absence of litter amendment. The relative abundance of AOA in our study (based on *16S rRNA* gene abundance) never exceeded 1% of the microbial community but was several fold higher than that of AOB and reflected previous results from other grasslands (16, 65, 66). The difference in abundance between AOA and AOB cannot on its own account for the orders of magnitude difference in transcription of *amoA*, suggesting that AOA were much more active than AOB in our soil. While slow growers, AOA can be highly metabolically active in soil (67). Moreover, transcription of archaeal *amoA* has also been shown to be correlated to nitrification rates (67–69). A similar experiment using a related species of *Avena* demonstrated that gross nitrification rates decreased with rhizosphere age (9). This pattern, which we also observed in *amoA* expression, could reflect increasing competition over the remaining labile N as well as reduced root exudation by the plant in an aging rhizosphere (70–72).

Expression of *amoA* has been previously shown to be dominated by few archaeal phylotypes (73–75). Not only do our results support this, but they quantitatively show that on average, 98% of the reads mapped to *amoA* were assigned to three variants, all archaeal. All three phylotypes were within the *Nitrososphaera* genus which is commonly found in soil (76). Similarly, *nirK* expression was also dominated by three variants, but surprisingly, these were most closely related to another soil AOA genus—*Nitrosocosmicus*. Additionally, expression of *amoA* and *nirK* was well correlated. It is possible that *Nitrososphaera* perform ammonium oxidation, with *Nitrosocosmicus* oxidizing the product. While in bacteria, these two processes are split between ammonium-oxidizing and nitrite-oxidizing bacteria, such a cooperation is not known in archaea. However, it is also possible that the taxonomic placement of one of the genes is incorrect. Both *amoA* and *nirK* have been identified in curated genomes of AOA (77–79), which raise the possibility that the same AOA cell can enact both steps of nitrification (80, 81), but dual expression of amoA and nirK by AOA has not yet been shown experimentally. It is also possible that the soil used here contains uncultured AOA that are far enough phylogenetically from known strains of *Nitrososphaera* and *Nitrosocosmicus* to cause a misassignment of *amoA* or *nirK* in a phylogenetic tree based on currently described representatives. The strong correlation between *amoA* and *nirK* suggests that there may be a relationship between these two processes, but further experimentation is required to determine if they are mechanistically linked like AOB and NOB or occur within the same cell.

Thaumarchaeal extracellular proteases identified in the same data set were previously found to be upregulated in bulk soil (3), and their temporal expression patterns highly correlated with *amoA* expression. *Thaumarchaeota* have been shown to prefer ammonium from organic nitrogen sources such as urea, amino acids, and peptides in forest soil (82). As AOA have to compete with the plant over ammonium in the rhizosphere, they may turn to organic N instead (83, 84); this could explain the tight correlation between *amoA* and AOA protease expression that we observed.

In our study, GS/GOGAT gene expression was widespread across treatments and over time, with significant upregulation in response to both roots and litter. Moreover, the expression of GS/GOGAT was consistently at least an order of magnitude higher than that of GDH. The first step of the GS/GOGAT pathway, *glnA* (glutamine synthetase), was one of the most highly expressed genes in our study. Ammonium assimilation via glutamate synthase (GS/GOGAT) is generally thought to be preferred to glutamate dehydrogenase under N limitation due to the K_m of GS/GOGAT which is an order of magnitude lower than that of GDH (85, 86). The P_{II} regulatory protein *glnB*, which is activated during N limitation and activates GS/GOGAT over GDH (87), was also highly expressed under all experimental conditions and upregulated in the presence of live or dead roots. In contrast, expression of the single-enzyme pathway of glutamate dehydrogenase was much lower. Assuming comparable post-transcriptional regulation, N limitation could drive a preference for multi-step unidirectional pathway GS/GOGAT (88) as opposed to the bidirectional GDH (89), as it prevents nitrogen loss from the organism (90). All these lines of evidence point toward N limitation in both rhizosphere and detritusphere in Mediterranean grassland soil.

Temporal effects of ammonium assimilation gene expression varied by gene, pathway, and habitat. Expression of *glnA* (GS/GOGAT) for ammonium assimilation was higher in the rhizosphere compared to bulk soil at 3, 6, and 12 days and was higher in litter-amended soil compared to unamended soil. Its expression was highest in litter-amended rhizosphere at 3 and 6 days, indicating there may be an additive effect of live roots growing through soil that contains root litter. This could be due to the high resource demand in a microhabitat where C is easily available via both litter and exudates; this may exacerbate cells' need to assimilate N to maintain their cellular stoichiometry (14, 91). In a prior analysis of this data set, we observed that carbohydrate active enzyme expression was also higher in the young rhizosphere, and expression of extracellular protease genes was highest at 3 days (2, 3). Thus, the additive effect of a combined rhizosphere-detritusphere appears to stimulate multiple pathways at once —carbohydrate depolymerization, organic N breakdown, and ammonium assimilation— reflecting synergistic breakdown and consumption processes (92). We note, however, that this effect may be short lived (93); in our data, it disappears by 6 days of rhizosphere maturation.

We also found that two GDH genes were upregulated in different habitats: rhizosphere (*gdh2*) and detritusphere (*gudB*). The expression of both genes decreased over time, possibly because ammonium concentrations dropped to a concentration too low for the affinity of these enzymes (86). The expression of *glnA* (GS/GOGAT) tells a more complicated story. In the litter-amended rhizosphere, its expression decreases over time, as does the expression of its regulatory genes. However, in the litter-amended bulk soil, the expression of *glnA* increases from day 6 on. The consistently lower expression of this enzyme in unamended bulk soil implies that any C input, be it from rhizodeposition of root litter, creates a demand for N that is at least somewhat alleviated via the GS/GOGAT pathway. Finally, we suspect there is a tradeoff between the GDH and GS/GOGAT pathways that corresponds to nitrogen limitation. Therefore, we examined the ratio of gene expression between *glnA* (GS/GOGAT) and *gudB*, the more highly expressed of the GDH genes. This ratio was consistently higher than 10, implying a general preference for GS/GOGAT, possibly due to low N availability at all times with or without the presence of roots or root litter. This ratio also increased over time under all experimental conditions, but this increase began earlier in the litter-amended rhizosphere. However, by 22

days, the highest ratio was in litter-amended bulk soil, possibly because growth rates are slower in bulk soil compared to the rhizosphere (54), and therefore, N limitation developed slower. This finding implies that the ratio of N assimilation pathways may be a useful proxy for the apparent N availability experienced by microorganisms—a useful metric in soils where inorganic N pools are consistently low but mineralization and assimilation fluxes are substantial (9, 94).

This study used an annual grass with a 12-week growth cycle, following transcripts for 3 weeks. It would be interesting to reproduce this experiment in a perennial grass in which the duration of the experiment would be longer, and competition between plant and microbes over N may be less temporally accentuated. Ideally, future studies would also include sensing nitrate, ammonium, and oxygen via microelectrodes in the rhizosphere and in bulk soil and gross rate estimates of nitrogen fluxes (e.g., using the ^{15}N pool dilution approach). We propose that the general mechanisms we have observed would be found in other grassland ecosystems, including perennials; however, the timescales would likely differ.

Conclusions

Most current knowledge of soil nitrogen cycling is based on measurements of bulk rates, soil enzyme assays, and genomic surveys and lacks insights on the spatial and temporal patterns that occur in distinct soil habitats. Here, we present a comprehensive analysis of gene expression of all major nitrogen cycling pathways over time in four common soil habitats: rhizosphere, litter-amended rhizosphere, litter-amended bulk soil, and unamended bulk soil of an annual grass. We propose several classes of controllers that shape the expression of nitrogen cycling genes in soil with or without roots and/or root litter, based on N availability, biotic competition, and the limitations of edaphic conditions. Expression of assimilation genes tended to be elevated in both detritusphere and rhizosphere soils, whereas dissimilatory processes such as denitrification and DNRA were upregulated primarily in the organic carbon-rich detritusphere. Processes that lead to loss of N from the system were downregulated near live roots. Availability of carbon and the quality of that C, whether from root exudates or root litter, appeared to drive N uptake to maintain cellular stoichiometry, and proximity to live roots likely led to competition for inorganic nitrogen. While gene expression levels may not necessarily predict protein abundance or enzyme activity, the use of metatranscriptomics allowed us to track multiple pathways and identify tradeoffs with fine-scale spatial and temporal resolution.

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DATA AVAILABILITY

The R project, necessary tables, N-cycling genes, DNA and protein sequences, tree files, and iTOL formatting files can be found at https://github.com/ellasiera/Ncycling. Raw metatranscriptome JGI accession numbers can be found in https://doi.org/10.1038/s41396-019-0582-x in Table S1.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental figures (mSystems00315-23-s0001.pdf). Figures S1-S3.
Supplemental tables (mSystems00315-23-s0002.xlsx). Tables S1-S3.

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