Transcriptome analysis reveals the regulatory mode by which NAA promotes the growth of Armillaria gallica

Jinlong Cai, Bilian Chen, Wenchao Li, Peng Xu, Yongguo Di, Huini Xu, Kunzhi Li

1 Faculty of Life Science and Technology, Kunming University of Science and Technology, Kunming, China,
2 Planting Department of Zhaoyang District Bureau of Agriculture, Zhaotong, China

* likzkm@163.com

Abstract

A symbiotic relationship is observed between Armillaria and the Chinese herbal medicine Gastrodia elata (G. elata). Armillaria is a nutrient source for the growth of G. elata, and its nutrient metabolism efficiency affects the growth and development of G. elata. Auxin has been reported to stimulate Armillaria species, but the molecular mechanism remains unknown. We found that naphthalene acetic acid (NAA) can also promote the growth of A. gallica. Moreover, we identified a total of 2071 differentially expressed genes (DEGs) by analyzing the transcriptome sequencing data of A. gallica at 5 and 10 hour of NAA treatment. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses showed that these unigenes were significantly enriched in the metabolism pathways of arginine, proline, propanoate, phenylalanine and tryptophan. The expression levels of the general amino acid permease (Gap), ammonium transporter (AMT), glutamate dehydrogenase (GDH), glutamine synthetase (GS), Zn(II) 2Cys6 and C2H2 transcription factor genes were upregulated. Our transcriptome analysis showed that the amino acid and nitrogen metabolism pathways in Armillaria were rapidly induced within hours after NAA treatment. These results provide valuable insights into the molecular mechanisms by which NAA promotes the growth of Armillaria species.

Introduction

Gastrodia elata (G. elata) is a valuable Chinese herbal medicine in China. This orchid species does not have roots and leaves, which can not absorb nutrients or perform photosynthesis. Thus, G. elata digests the symbiotic Armillaria mellea (A. mellea) to provide its nutrition [1–3]. A. mellea with a high growth rate can promote the growth of G. elata. Therefore, many studies have focused on promoting the growth of A. mellea. Screening tests for woody habitats suitable for the growth of A. mellea showed that white sandalwood, oak and walnut significantly promoted the growth of A. mellea while lilac inhibited the growth [4, 5]. Research on providing supplementary nutrition to A. mellea also showed that potato and carrot were beneficial to its growth [6]. Plant growth regulators, such as NAA [7], indole-3-acetic acid [8],
2,4-D [9], tannins [10], triacontanol and inositol [11], can regulate the growth of *A. mellea*. Promoting the growth of *A. mellea* has become an important field of *G. elata* research.

Fungi can provide plants with mineral nutrients. Arbuscular mycorrhiza (AM) represents a symbiotic association between a fungus (*Glomeromycota* spp.) and the roots of plant species [12–14]. The establishment of symbiosis relies on nutrient exchange between mycorrhizal fungi and plants. Nitrogen and phosphorus are the basic mineral nutrients for plant growth, and certain fungal species can promote plant uptake of mineral elements in soil, such as nitrogen and phosphorus [15, 16], and help plants absorb water and other trace mineral elements. The nutrient exchange between AM fungi and plants is the basis for maintaining their symbiotic relationship. Plants provide AM fungi with a carbon source for their growth [17–19], and in exchange, AM fungi provide plants with mineral nutrients, mainly phosphorus and nitrogen [20, 21]. Transporters in mycelia can absorb nitrogen nutrients, such as inorganic nitrogen and amino acids. At present, AMT genes from different mycorrhizal fungi have been isolated, and these transporter genes include *AMT1*, *AMT2* and *AMT3*, which belong to the *Mep/Amt* gene family [22, 23]. In yeast [24] and filamentous fungi [25, 26], amino acid transporters have been extensively and deeply studied. Recently, Cappellazzo et al. [27] isolated the amino acid transporter gene *Gmos AAP1* from AM fungi. Extraroot hyphae take up NH$_4^+$ and NO$_3^-$ from soil through AMT and nitrate transporters, respectively [28]. In mycelia, AM fungi reduce NO$_3^-$ to NH$_4^+$ through reductase. Then, glutamine synthetase synthesizes NH$_4^+$ and glutamic acid provided by plants into glutamine [29]. Through a series of biosyntheses, glutamine is converted into arginine [30], which is transported within the mycelium. Finally, arginine is degraded into NH$_4^+$ by the urea cycle, and NH$_4^+$ is then transferred into the exosome of the plant cytoplasmic membrane by a nitrogen transport ion pump and absorbed by the plant [31].

Although the establishment of symbiosis relies on nutrient exchange between mycorrhizal fungi and plants, the mechanism underlying arbuscular mycorrhiza formation remains poorly understood. Recent reports suggested that certain plant hormones are also important for arbuscule development. Abscisic acid, gibberellin acid and strigolactones have an important function in arbuscule maintenance and formation [32–35]. Although the mechanism of auxin in this type of plant–microbe interaction is unclear, studies have shown that auxin also plays a role in AM symbiosis [36–38]. The content of auxin varies in different mycorrhizal roots, with its content remaining stable in tobacco and leek mycorrhiza [39, 40] but increasing in mycorrhizal maize and soybean roots [41–43]. In the mycorrhizal roots of mutant nark soybeans with defects in the automatic regulation of nodulation, the increase in IAA content was low, indicating that IAA may have a function in the automatic regulation of mycorrhization [42]. Recent studies have found that auxin could play a role in AM colonization [36, 44]. Auxin perception and/or auxin signaling are important for arbuscule development [45, 46]. When cultured on medium supplemented with auxin, *A. mellea* grew vigorously and produced abundant rhizomorphs [47]. In culture, 2,4-dichlorophenoxyacetic acid and NAA could stimulate the growth rate of rhizomorphs of *A. mellea* [7, 9, 48]. Although NAA can promote the growth of *Armillaria*, its mechanism remains unclear.

In this study, we used RNA-seq analysis to determine the mechanism by which NAA promotes growth. Based on a DEG analysis, we proposed a hypothetical regulatory network of NAA that promotes the growth of *A. gallica*. In *A. gallica*, NAA promoted the expression of transcription factors, which in turn upregulated the expression levels of AMT and Gap genes. Then, the transcription levels of glutamate dehydrogenase and glutamine synthase genes were increased to promote amino acid and nitrogen metabolism in *A. gallica*, thereby promoting its growth. This hypothetical regulatory network might provide a theoretical basis for further studies on the molecular mechanism by which NAA promotes the growth of *A. gallica*. 
Materials and methods

Culture of A. gallica

A. gallica strain AG01 was isolated from G. elata f. glauca in Zhao tong. To assess the ability of NAA to promote the growth of A. gallica, fungal cultures were prepared by adding 50 mL of melted (60˚C) semisolid PDA (for 1 L PDA: 200 g peeled potatoes, 20 g dextrose, 2 g agar) medium to sterile tissue culture bottles containing NAA (8 mg/L) or solvent control. The inoculum used to seed the medium was a 0.5 cm tip of the rhizomorph. The fungi were incubated in the dark at 25˚C. The rhizomorphs were separated from the media, and the dry weights were determined after 6, 12 and 18 days of growth. The results are based on one representative of three independent experiments.

Cultivated material was used to study the early response mechanism of A. gallica to NAA. The strain of A. gallica was inoculated in inclined tubes filled with PDA medium and cultured at 25˚C for 10 days. When the mycelium had filled the inclined plane, it was transferred to liquid culture medium [49]. Then, the culture was shaken for 7 to 10 days at 115 rpm and 25˚C. Based on previous experiments (S1 Fig), mycelium was collected immediately after 5 h and 10 h of NAA treatment, frozen in liquid nitrogen and stored at −80˚C for further analysis.

RNA extraction, library construction, and transcriptome sequencing

Total RNA was extracted with the RNAprep Pure Plant Kit (TIANGEN, China) and then quantified using a Nanodrop2000 spectrophotometer (NanoDrop Technologies, USA) and an Agilent 2100 Bioanalyzer (Agilent, USA).

For Illumina RNA sequencing, sequencing libraries were generated from the total RNA samples with the NEBNext Ultra™ II RNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer’s recommendations. The cDNA libraries were sequenced on an Illumina HiSeq 2000 platform (Illumina, USA).

Quantification of gene expression levels

Gene expression levels were estimated by RSEM [50] for each sample as follows:
1. Clean data were mapped back onto the assembled transcriptome;
2. Read counts for each gene were obtained from the mapping results.

Differential expression analysis

Differential expression analyses of pairs of groups were performed using the DESeq R package (1.10.1). The P values were adjusted using Benjamini and Hochberg’s approach for controlling the false discovery rate. Genes with an adjusted P value <0.05 found by DESeq were considered differentially expressed.

GO and KEGG enrichment analysis of DEGs

Gene Ontology (GO) enrichment analyses of the differentially expressed genes (DEGs) were implemented by the top GO R package-based Kolmogorov–Smirnov test. We used KOBAS software [51] to test the statistical enrichment of differentially expressed genes in KEGG pathways.

Quantitative PCR analysis

Total RNA was extracted from mycelium using RNAiso Plus (TaKaRa) according to the manufacturer’s recommended protocols. One microgram of total RNA was reverse-transcribed
into first-strand cDNA with oligo dT primers using HiScript® II Reverse Transcriptase for RT–qPCR (Vazyme) following the manufacturer’s instructions, and cDNA templates were stored at −20°C until use. The RT–qPCR protocol consisted of an initial heat activation step of 95°C for 30 s, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Three biological replicates were performed for each treatment, and each biological replicate consisted of three technical replicates.

The relative expression levels of the target genes were calculated using the $2^{-\Delta\Delta CT}$ approach, with normalization of data to the geometric average of two reference control genes [52].

Statistical analysis

Statistical analyses were performed using SPSS statistic 22.0 software. Duncan’s multiple test ($P < 0.05$) was chosen for statistical analysis. Data are the means ± SE from at least three independent biological replicates.

Results

NAA promoted the growth of A. gallica

We used 8 mg/L NAA to assess the effect of auxin treatment on the growth of A. gallica. On the medium with NAA, A. gallica grew profusely and consisted of many rhizomorphs (Fig 1A). At this NAA concentration, the dry weight of mycelium was significantly higher than that of the control after 6 and 12 days of A. gallica growth. However, significant differences from that of the control were not observed after 18 days of A. gallica growth (Fig 1B).

Transcriptomic analysis of A. gallica mycelium in response to NAA at different time points

To analyze the genes that may participate in NAA-promoted A. gallica mycelium growth, differences in gene expression were examined in the mycelium of A. gallica treated with NAA for 5 and 10 h. A total of 2071 DEGs were identified in A. gallica mycelium, 813 at 5 h and 1258 at 10 h, respectively (Fig 2A). The distribution of up- and downregulated genes was counted for each time point and is shown in a Venn diagram (Fig 2B). After treatment with NAA for 5 and 10 h, a group of unique genes were upregulated (total 812), with 150 genes significantly upregulated at both time points. In addition, a number of genes were significantly downregulated (total 1003), with 106 genes showing reduced expression at both time points.

Furthermore, a heatmap (Fig 2C) was generated to provide an overview of the gene expression changes and expression pattern. The expression patterns of most DEGs subjected to the NAA treatment showed opposite trends at 5 h and 10 h (Fig 2C). Most of the genes with lower expression levels at 5 h had higher expression levels at 10 h, and vice versa. Compared with the CK (without NAA), the expression profiles of most DEGs under the NAA treatments showed great differences.

Functional classification of the DEGs by GO and KEGG pathway analysis

To identify NAA-induced genes, GO and KEGG pathway analyses were used to functionally classify the DEGs. In the GO analysis, the DEGs induced by NAA were classified into three main GO categories (Fig 3). At 5 h and 10 h (Fig 3), the DEGs in the biological process category were significantly enriched in metabolic processes, cellular processes and single-organism processes. The majority of DEGs in the molecular function category were enriched in catalytic activity and binding, although differences in molecular functions were observed. Only downregulated DEGs were enriched in molecular transducer activity and signal transducer activity,
while only upregulated DEGs were also enriched in nutrient reservoir activity. The GO pathway analyses indicated that NAA promotes the metabolism of certain nutrients but inhibits the metabolism of others by acting on signaling pathways.

To further reveal the biological function of NAA-induced DEGs, we performed enrichment analyses based on the KEGG database. The top 20 pathways for the most prominent DEGs were identified (Fig 4). The downregulated DEGs were mostly enriched in “sesquiterpenoid and triterpenoid biosynthesis”, “cell cycle-yeast”, “one carbon pool by folate”, “methane 

Fig 1. Effect of NAA on the growth of *A. gallica*. (A) Morphology of *A. gallica* after 6, 12 and 18 days of growth. *A. gallica* were cultured on medium with NAA or without NAA (CK). Scale bar = 1 cm. (B) Dry weights of *A. gallica* in different culture times. The values are the means ± SE of three biological replicates. Asterisks indicate significant differences (* p < 0.05, ANOVA). 

https://doi.org/10.1371/journal.pone.0277701.g001
metabolism”, “steroid biosynthesis” and “tryptophan metabolism” (Fig 4A), while the upregulated DEGs were significantly enriched in “arginine and proline metabolism”, “fatty acid biosynthesis”, “propanoate metabolism”, “phenylalanine metabolism”, and “ascorbate and aldarate metabolism”. In particular, “tryptophan metabolism” was significantly enriched in the KEGG pathway analysis (Fig 4B). Transcriptome analysis showed that NAA positively affected
amino acid metabolism in *A. gallica* and the transcript levels of nitrogen metabolism-associated genes.

In our current study, the transcript levels of 34 amino acid metabolism- and 4 nitrogen metabolism-related structural genes were analyzed, and the expression levels of those genes (except the nitrite reductase gene) were upregulated under NAA treatment (Table 1). Thirty-four putative ATM (amino acid transport and metabolism) genes were identified, among which the expression levels of 14 putative ATM genes were upregulated with NAA treatment at both time points. The transcript levels of 12 putative ATM genes were upregulated (1.02- to 1.38-fold) with NAA treatment at 10 h. Additionally, the other putative ATM genes in *A. gallica* were significantly induced (1.18- to 4.89-fold) in response to NAA treatment at both time points (Table 1). AMT, GS and GDH genes, which play an important role in nitrogen

---

**Table 1.** DEGs associated with amino acid and nitrogen metabolism in *A. gallica* in response to NAA treatment.

| Gene Description                     | Gene ID | Nr_annotation | Log2Fold Change 5 h | Log2Fold Change 10 h |
|--------------------------------------|---------|----------------|---------------------|----------------------|
| Zn(II)2Cys6 transcription factor     | c26769.graph_c0 | Hypothetical protein ARMGADRAFT_955812 | 1.04 /               |                       |
|                                      | c18863.graph_c0 | hypothetical protein ARMSODRAFT_1090953 | 1.02 1.73         |                       |
|                                      | c24787.graph_c0 | uncharacterized protein ARMOST_00211 | 1.08 /               |                       |
|                                      | c27966.graph_c0 | hypothetical protein ARMGADRAFT_638842 | 1.21 /               |                       |
|                                      | c23323.graph_c0 | hypothetical protein ARMSODRAFT_223645 | 1.15 /               |                       |
|                                      | c15606.graph_c0 | hypothetical protein ARMGADRAFT_1063831 | 1.03 /               |                       |
|                                      | c24507.graph_c0 | TPT-domain-containing protein | 1.42 /               |                       |
|                                      | c23156.graph_c0 | hypothetical protein ARMGADRAFT_1071141 | 1.23 /               |                       |
| C2H2 Zin finger proteins             | c26198.graph_c0 | STE-domain-containing protein | 1.06 /               |                       |
|                                      | c24508.graph_c1 | hypothetical protein ARMGADRAFT_1010957 | 1.16 /               |                       |
|                                      | c24448.graph_c0 | hypothetical protein ARMGADRAFT_1074413 | / 1.33              |                       |
|                                      | c27199.graph_c0 | uncharacterized protein ARMOST_04702 | / 1.53              |                       |
|                                      | c10985.graph_c0 | hypothetical protein ARMGADRAFT_1014537 | 1.11 /               |                       |
|                                      | c16248.graph_c0 | hypothetical protein ARMGADRAFT_997381 | / 2.00               |                       |
|                                      | c9941.graph_c0 | hypothetical protein ARMGADRAFT_1065654 | / 1.91               |                       |

(Continued)
metabolism, were identified, and they displayed similar expression patterns under NAA treatment, with upregulation at 10 h, including c19578.graph_c0, c21900.graph_c0 and c24003.graph_c0.

| Gene Description                                      | Gene ID          | Nr_annotation                              | Log2Fold Change 10 h |
|-------------------------------------------------------|------------------|--------------------------------------------|---------------------|
| Amino acid transport and metabolism                   | c26398.graph_c0  | high affinity methionine permease          | 1.44 1.89           |
|                                                       | c23593.graph_c0  | hypothetical protein ARMGADRAFT_1058748   | 1.23 /              |
|                                                       | c19166.graph_c0  | vacuolar amino acid permease               | 1.15 /              |
|                                                       | c23409.graph_c0  | related to uracil permease                 | 1.36 /              |
|                                                       | c26755.graph_c0  | amino-acid permease inidal                  | / 1.27              |
|                                                       | c19079.graph_c0  | hypothetical protein ARMGADRAFT_1171443    | / 1.2               |
|                                                       | c26291.graph_c0  | MFS general substrate transporter          | / 1.02              |
|                                                       | c25774.graph_c0  | hypothetical protein ARMGADRAFT_1078564    | / 1.36              |
|                                                       | c26100.graph_c0  | DAO-domain-containing protein              | 1.02 /              |
|                                                       | c20438.graph_c0  | PLP-dependent transferase                   | 1.65 /              |
|                                                       | c10068.graph_c0  | hypothetical protein ARMGADRAFT_1004296    | 1.42 2.06           |
|                                                       | c17479.graph_c0  | hypothetical protein ARMGADRAFT_1036273    | 1.09 /              |
|                                                       | c22013.graph_c0  | aryl-alcohol oxidase precursor             | 1.98 /              |
|                                                       | c22406.graph_c0  | PLP-dependent transferase                   | / 1.38              |
|                                                       | c23177.graph_c0  | Clavaminic synthase-like protein            | / 1.07              |
|                                                       | c23191.graph_c0  | Dehydrogenase ARMGADRAFT_1018426           | 2.05 /              |
|                                                       | c23517.graph_c0  | aryl-alcohol oxidase-like protein           | 1.20 /              |
|                                                       | c23882.graph_c0  | alcohol oxidase                            | 1.52 1.94           |
|                                                       | c23917.graph_c0  | alpha/beta-hydrolase                       | / 1.32              |
|                                                       | c24128.graph_c0  | Homocysteine S-methyltransferase           | / 1.10              |
|                                                       | c24915.graph_c0  | aryl-alcohol oxidase precursor             | 2.94 1.70           |
|                                                       | c25006.graph_c1  | pyranose dehydrogenase                     | / 1.06              |
|                                                       | c25006.graph_c2  | pyranose dehydrogenase                     | / 1.31              |
|                                                       | c25251.graph_c0  | related to Tyrosinase                      | 1.03 /              |
|                                                       | c25695.graph_c0  | hypothetical protein ARMGADRAFT_735571     | 1.04 1.57           |
|                                                       | c26168.graph_c0  | NPD-domain-containing protein              | 1.18 /              |
|                                                       | c26688.graph_c0  | MATE efflux family protein                 | / 1.35              |
|                                                       | c26823.graph_c0  | glutaryl-CoA dehydrogenase                 | 1.30 1.18           |
|                                                       | c27283.graph_c0  | hypothetical protein ARMGADRAFT_1033881    | 1.98 4.89           |
|                                                       | c9872.graph_c0   | acetylornithine aminotransferase, partial   | / 1.31              |
|                                                       | c27161.graph_c1  | uncharacterized protein ARMOST_19843       | 1.21 /              |
|                                                       | c27349.graph_c0  | alcohol oxidase                            | 2.73 1.41           |
|                                                       | c27955.graph_c0  | hypothetical protein ARMSODRAFT_942066     | 1.49 /              |
|                                                       | c28019.graph_c0  | aryl-alcohol oxidase precursor             | 1.89 /              |
| Nitrile reductase                                      | c17687.graph_c0  | FAD/NAD(P)-binding domain-containing protein| / -1.74             |
| Ammonium transporter                                   | c19578.graph_c0  | ammonium transporter                       | / 1.93              |
| Glutamine synthetase                                  | c21900.graph_c0  | related to RPL3-60s ribosomal protein I3    | / 1.00              |
| Glutamate dehydrogenase                               | c24003.graph_c0  | NADP-specific glutamate dehydrogenase      | / 1.42              |

"/" indicates no significant differences between the NAA treatment groups and CK.

https://doi.org/10.1371/journal.pone.0277701.t001
Expression profiling of transcription factors associated with amino acid metabolism and nitrogen metabolism

Transcription factors were significantly induced in response to NAA treatment, and they might play important regulatory roles in amino acid metabolism and nitrogen metabolism. In this study, a total of 21 transcription factors were identified as putative regulators of amino acid metabolism and nitrogen metabolism in response to NAA treatment. They included Zn(II)$_2$Cys$_6$ (Zn2 Cys6 Zn clusters) and C2H2s (C2H2 zinc-finger proteins) (Table 1). The C2H2s might be the main determinant of amino acid and nitrogen metabolism in response to NAA in $A$. gallica because C2H2s accounted for the largest percentage. All 9 of the putative Zn(I)$_2$Cys$_6$ TFs were upregulated under NAA treatment at 5 h. Among them, seven genes showed no change at 10 h while the other two genes were still significantly upregulated (Table 1). In this study, 12 putative C2H2 genes were identified, and most were upregulated under NAA treatment (Table 1). Furthermore, the expression levels of 7 putative C2H2s increased under NAA treatment at 5 h and the other 5 putative C2H2 genes showed upregulation at 10 h. These findings suggest that the above transcription factors might be involved in regulating NAA-promoted amino acid and nitrogen metabolism.

qRT–PCR validation of differentially expressed genes was performed to validate whether the RNA-seq data truly reflected the actual transcription level. We selected 9 genes for quantitative real-time PCR (polymerase chain reaction) to detect DEG expression levels at 5 and 10 h (Fig 5). These genes included transcription factor genes and amino acid and nitrogen metabolism genes. In the qRT–PCR analysis, the expression patterns of these genes were very similar to the FPKM values from sequencing under the corresponding treatment, indicating that the RNA-seq data are reliable.

Discussion

A symbiotic relationship is observed between $A$. gallica and $G$. elata, which is a traditional Chinese medicinal plant that obtains the nutrients needed for growth and development from $A$. gallica, which obtains nutrients by decomposing wood. To protect the environment and improve the yield of $G$. elata, the utilization efficiency of nutrients by $A$. gallica must be improved. A number of secondary metabolites play an important role in plant nutrient acquisition and abiotic stress tolerance. For example, under iron deficiency conditions, Arabidopsis relies on coumarin secretion to change the root microbial community [53]. The bacterial root
microbiota, which is stimulated by secreted coumarins, promotes adaptions to iron-limited soil conditions [54]. IAA is involved in the establishment of biotrophy in *Piriformospora indica*-barley symbiosis [55]. IAA can promote fungal invasion and AM formation, especially at early stages [56]. Our findings were consistent with previous reports showing that NAA can also promote the growth and biomass of *Armillaria* [7, 47, 57]. Under NAA treatment, the dry weight of mycelium was significantly higher than that of the control after 6 and 12 days of growth (Fig 1). However, significant differences were not observed after 18 days (Fig 1B), which may be due to the continuous consumption of NAA as *A. gallica* grows until an ineffective concentration was reached at 18 days [7, 9, 48]. Auxin signal components have been suggested to be important nitrogen (N)-responsive regulators of root architecture. For example, in response to external N, mutants lacking ARF8 or AFB3 showed compromised root development [58, 59]. In *Arabidopsis*, auxin can be transported by the dual-affinity NO$_3^-$ transporter NRT1.1 [60] and plays a major role in lateral root emergence and growth induced by low N availability [61]. A recent study showed that the accumulation of auxin enhanced NO$_3^-$ uptake and assimilation. Auxin response factors promote N-use efficiency and grain yield by transactivating the expression of genes related to NO$_3^-$ metabolism [62]. These papers proved that auxin has beneficial biological effects in the nitrogen response. In the current study, we discovered that the expression of most genes associated with amino acid and nitrogen metabolism was upregulated by NAA (Table 1), including the genes encoding Gap, AMT, GDH and GS. However, the expression of the nitrite reductase gene was downregulated at 10 h, which might be related to the lack of nitrate nitrogen in the medium [49]. This study expands upon the knowledge of the molecular mechanisms underlying the ability of NAA to promote the growth of *Armillaria*.

Fig 6. Speculative model of NAA promoting amino acid and nitrogen metabolism in *A. gallica*. Under NAA treatment, the expression of transcription factor genes was upregulated. Then, transcription factors promote the expression of amino acid and ammonium transporter genes. It promotes the expression of other amino acid and nitrogen metabolism related genes, thereby promoting amino acid and nitrogen metabolism.

https://doi.org/10.1371/journal.pone.0277701.g006
NAA may promote nitrogen use efficiency and Armillaria growth by activating the expression of genes related to nitrogen and amino acid metabolism. Auxin response factors increase nitrogen use efficiency by promoting the expression of genes related to NO$_3^-$ metabolism [62]. Similarly, we found that NAA significantly promoted the expression levels of genes related to amino acid and nitrogen metabolism pathways. Moreover, the expression levels of 34 putative genes of ATM were upregulated under NAA treatment. With increasing NAA treatment time, the expression levels of AMT, GS and GDH, which play an important role in nitrogen metabolism, were all upregulated. AM fungi can take up NH$_4^+$ in soil through AMT [28] and then synthesize glutamine from NH$_4^+$ and glutamic acid through glutamine synthetase [29]. In this study, the expression levels of GHS and GS genes were increased, which may be related to the ability of AMT and ATM to transport NH$_4^+$ and amino acids into cells, respectively, resulting in increased NH$_4^+$ and amino acid contents in cells, which in turn promote the expression of GS and GHS.

Many researchers have extensively characterized transcription factors that regulate the expression of AMT and Gap genes in fungi. The transcription of the genes encoding ammonium permease and Gap was shown to be dependent on the transcription factor Gln3 in Candida glabrata [63]. Under low ammonium conditions, the deletion of the AREA transcription factor led to a significant reduction in the expression of the three predicted ammonium permease genes [64]. In rice, auxin-mediated promotion of NO$_3^-$ uptake is controlled by members of the OsARF family, such as OsARF6 and OsARF17, which synergistically promote NO$_3^-$ metabolism [62]. In this study, 21 transcription factors that responded to the NAA treatment were identified as putative regulators of amino acid and nitrogen metabolism, including Zn(II) 2Cys6-encoding genes and C2H2-encoding genes (Table 1). Not only did the expression level of amino acid and nitrogen metabolism-related genes change with NAA treatment time, but the TF genes also had variable expression levels. This finding suggests that these TFs are likely the main regulators of amino acid and nitrogen metabolism-related genes in A. gallica under NAA treatment.

In our study, the expression levels of most of the studied transcription factors were increased under NAA. This finding suggests that the regulatory role of TFs may play an important role in NAA promoting amino acid and nitrogen metabolism. At present, NAA has been shown to promote the growth of Armillaria; however, the molecular mechanism underlying the ability of NAA to promote the growth of Armillaria has not been elucidated. Based on this transcriptome analysis and previous studies, a putative regulatory network was proposed whereby NAA stimulated amino acid and nitrogen metabolism to promote A. gallica growth (Fig 6). Under the NAA treatment, transcription factor gene expression was upregulated, which then regulated the transcription of amino acid and nitrogen metabolism-related genes. These changes may at least partially explain why the biomass of A. gallica increased under the action of NAA. Further studies should be carried out to better understand the mechanism by which NAA promotes the growth of A. gallica.

**Conclusion**

This study found that 8 mg/L NAA can promote the growth of A. gallica, and an analysis of the transcriptome sequencing data of A. gallica identified a total of 2071 DEGs. GO and KEGG pathway enrichment analyses revealed that most of the DEGs were involved in amino acid and nitrogen metabolism under NAA treatment. We also found that the expression levels of genes encoding Gap, AMT, GDH and GS were upregulated. Zn(II)2Cys6 and C2H2 are putative transcription factors related to amino acid and nitrogen metabolism, and they were also identified. This finding revealed that amino acid and nitrogen metabolism-related genes would be
rapidly activated by NAA. This study may accelerate the process of revealing the regulatory mechanisms by which NAA promotes the growth of *Armillaria*.

**Supporting information**

**S1 Fig.** Expression of glutamine synthetase (GS), glutamate dehydrogenase (GDH) and amino-acid permease inda1 (AAP) genes of *A. gallica* after NAA treatment at 2, 4, 5, 6 and 8 h. The y-axis represents the relative gene expression levels analyzed by qRT–PCR. The values are the means ± SE of three biological replicates. Statistically significant differences are indicated by letters above columns (*P* < 0.05, ANOVA).

(DOCX)

**Author Contributions**

**Conceptualization:** Bilian Chen.

**Data curation:** Bilian Chen, Wenchao Li.

**Formal analysis:** Huini Xu.

**Funding acquisition:** Kunzhi Li.

**Investigation:** Wenchao Li, Peng Xu.

**Methodology:** Jinlong Cai.

**Resources:** Yongguo Di.

**Supervision:** Huini Xu.

**Writing – original draft:** Jinlong Cai.

**Writing – review & editing:** Kunzhi Li.

**References**

1. Kikuchi G, Yamaji H. Identification of *Armillaria* species associated with *Polyporus umbellatus* using ITS sequences of nuclear ribosomal DNA. Mycoscience. 2010; 51: 366–372.
2. Guo T, Wang HC, Xue WQ, Zhao J, Yang ZL. Phylogenetic analyses of *Armillaria* reveal at least 15 phylogenetic lineages in China, seven of which are associated with cultivated *Gastrodia elata*. PLOS One. 2016; 11: e0154794.
3. Xing YM, Li B, Liu L, Li Y, Yin SX, Yin SC, et al. *Armillaria mellea* symbiosis drives metabolomic and transcriptomic changes in *Polyporus umbellatus* Sclerotia. Front Microbiol. 2022; 12: 1–9.
4. Huang WJ, Wu XB, Wang QY. Effect of different geo-authentic trees in southwestern Yunnan on *Armillaria mellea* cultivation. Mod Chin Med. 2015; 17: 475–478. (Chinese)
5. Wang QY, Li LT. Study on the *Armillaria mellea* cultured wood of eight kinds of trees in Tengchong country of Yunnan Province. Mod Chin Med. 2016; 18: 888–890. (Chinese)
6. Ao XY, Peng SM, Chen YuH, Effect of supplementary nutrient sources on the growth of the cultivation seeds of *Armillaria mellea*. Chinese Agricultural Science Bulletin. 2012; 28: 212–216. (Chinese)
7. Yang WH, Huang MJ, Huang HQ. Effect of NAA on growth of *Armillaria mellea* of *Gastrodia elata* Blume in Zhaotong. Chinese Agricultural Science Bulletin. 2013; 09: 169–171. (Chinese)
8. Garraway MO. Rhizomorph initiation and growth in *Armillaria mellea* promoted by o-aminobenzoic and p-aminobenzoic acids. Phytopathology. 1970; 60: 861–865.
9. Pronos J, Patton RF. The effect of chlorophenoxy acid herbicides on growth and rhizomorph production of *Armillariella mellea*. Phytopathology. 1979; 69: 136–141.
10. Brazee NJ, Wick RL, Wargo PM. Effects of hydrolyzable tannins on in vitro growth of *Armillaria calvescens* and *A. gallica*. Plant Dis. 2011; 95: 1255–1262.
11. Zhou JS, Jiao YC, Sheng HY, Xiong HY, Sheng HY. Effects of triacontanol, pH of culture medium and culture temperature on *Armillaria lutea*. Edible fungi. 2011; 33:8–9. (Chinese)
12. Davison J, Moora M, Opik M, Adholeya A, Ainsaar L, Bä A, et al. FUNGAL SYMBIONTS. Global assessment of arbuscular mycorrhizal fungus diversity reveals very low endemism. Science. 2015; 349: 970–973. https://doi.org/10.1126/science.aab1161 PMID: 26315436

13. Zhang S, Lehmann A, Zheng W, You Z, Rillig MC. Arbuscular mycorrhizal fungi increase grain yields: a meta-analysis. New Phytol. 2019; 222: 543–555. https://doi.org/10.1111/nph.15570 PMID: 30372522

14. van’t Padje A, Werner GDA, Kiers ET. Mycorrhizal fungi control phosphorus value in trade symbiosis with host roots when exposed to abrupt “crashes” and “booms” of resource availability. New Phytologist. 2021; 229: 2933–2944. https://doi.org/10.1111/nph.17055 PMID: 33124078

15. Smith FA, Smith SE. What is the significance of the arbuscular mycorrhizal colonisation of many economically important crop plants? Plant Soil. 2011; 348: 63–79.

16. Müller LM, Harrison MJ. Phytohormones, miRNAs, and peptide signals integrate plant phosphorus status with arbuscular mycorrhizal symbiosis. Curr Opin Plant Biol. 2019; 50: 132–139. https://doi.org/10.1016/j.pbi.2019.05.004 PMID: 31212139

17. Jiang Y, Wang W, Xie Q, Liu N, Liu L, Wang D, et al. Plants transfer lipids to sustain colonization by mutualistic mycorrhizal and parasitic fungi. Science. 2017; 356: 1172–1175. https://doi.org/10.1126/science.aam9970 PMID: 28596307

18. Luginbuehl LH, Manerd GN, Kurup S, Van Erp H, Radhakrishnan GV, Breakspear A, et al. Fatty acids in arbuscular mycorrhizal fungi are synthesized by the host plant. Science. 2017; 356: 1175–1178. https://doi.org/10.1126/science.aan0081 PMID: 28596311

19. Charters MD, Sait SM, Field KJ. Aphid herbivory drives asymmetry in carbon for nutrient exchange between plants and an arbuscular mycorrhizal fungus. Curr Biol. 2020; 30: 1801–1808. https://doi.org/10.1016/j.cub.2020.02.087 PMID: 32275877

20. Leigh J, Hodge A, Fitter AH. Arbuscular mycorrhizal fungi can transfer substantial amounts of nitrogen to their host plant from organic material. New Phytol. 2009; 181: 199–207. https://doi.org/10.1111/j.1469-8137.2008.02630.x PMID: 18811615

21. Tedersoo L, Bahram M. Mycorrhizal types differ in ecophysiology and alter plant nutrition and soil processes. Biological Reviews. 2019; 94: 1857–1880. https://doi.org/10.1111/brv.12538 PMID: 31270944

22. Javelle A, Morel M, Rodríguez-Pastrana BR, Botton B, André B, Marin AM, et al. Molecular characterization, function and regulation of ammonium transporters (Amt) and ammonium-metabolizing enzymes (GS, NADP-GDH) in the ectomycorrhizal fungus *Hebeloma cylindrosporum*. Mol Microbiol. 2003; 47: 411–430.

23. Willmann A, Weiss M, Nehls U. Ectomycorrhiza-mediated repression of the high-affinity ammonium importer gene *AmAMT2* in *Amanita muscaria*. Curr Genet. 2007; 51: 71–78.

24. Bianchi F, van’t Klooster JS, Ruiz SJ, Poolman B. Regulation of amino acid transport in *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev. 2019; 83: e00024–19.

25. Struck C, Ernst M, Hahn M. Characterization of a developmentally regulated amino acid transporter (AAT1p) of the rust fungus *Uromyces fabae*. Mol Plant Pathol. 2002; 3: 23–30.

26. Trip H, Evers ME, Konings WN, Driessen AJ. Cloning and characterization of an aromatic amino acid importer gene in *Penicillium chrysogenum*. Biochim Biophys Acta. 2002; 1565: 73–80.

27. Cappellazzo G, Lanfranco L, Fitz M, Wipf D, Bonfante P. Characterization of an amino acid permease from the endomycorrhizal fungus *Glomus mosseae*. Plant Physiol. 2008; 147: 429–437.

28. Ashford AE, Allaway WG. The role of the motile tubular vacuole system in mycorrhizal fungi. Plant and Soil. 2002; 244: 177–187.

29. Breuninger M, Trujillo CG, Serrano E, Fischer R, Requena N. Different nitrogen sources modulate activity but not expression of glutamine synthetase in arbuscular mycorrhizal fungi. Fungal Genet Biol. 2004; 41: 542–552. https://doi.org/10.1016/j.fgb.2004.01.003 PMID: 15050543

30. Fellbaum CR, Gachomo EW, Beeetsey Y, Choudhari S, Strahahn GD, Pfeffer PE, et al. Carbon availability triggers fungal nitrogen uptake and transport in arbuscular mycorrhizal symbiosis. Proc Natl Acad Sci U S A. 2012; 109: 2666–2671. https://doi.org/10.1073/pnas.1118650109 PMID: 22306426

31. Govindaraju M, Pfeffer PE, Jin H, Abubaker J, Douds DD, Allen JW, et al. Nitrogen transfer in the arbuscular mycorrhizal symbiosis. Nature. 2005; 435: 819–823. https://doi.org/10.1038/nature03610 PMID: 15944705

32. Herrera-Medina MJ, Steinkellner S, Vierheilig H, Ocampo Bote JA, García Garrido JM. Abscisic acid determines arbuscule development and functionality in the tomato arbuscular mycorrhiza. New Phytol. 2007; 175: 554–564. https://doi.org/10.1111/j.1469-8137.2007.02107.x PMID: 17633230

33. Floss DS, Levy JG, Lévesque-Tremblay V, Pumplin N, Harrison MJ. DELLA proteins regulate arbuscule formation in arbuscular mycorrhizal symbiosis. Proc Natl Acad Sci USA. 2013; 110: E5025–E5034. https://doi.org/10.1073/pnas.1308973110 PMID: 24297892
34. Foo E, Ross JJ, Jones WT, Reid JB. Plant hormones in arbuscular mycorrhizal symbioses: an emerging role for gibberellins. Ann Bot. 2013; 111: 769–779. https://doi.org/10.1093/aob/mct041 PMID: 23508650

35. Aquino B, Bradley JM, Lumba S. On the outside looking in: roles of endogenous and exogenous strigolactones. Plant J. 2021; 105: 322–334. https://doi.org/10.1111/tpj.15087 PMID: 33215770

36. Hanlon MT, Coenen C. Genetic evidence for auxin involvement in arbuscular mycorrhiza initiation. New Phytol. 2011; 189: 701–709. https://doi.org/10.1111/j.1469-8137.2010.03567.x PMID: 21091696

37. Liao DH, Wang SS, Cui MM, Liu J, Chen A, Xu G. Phytohormones regulate the development of arbuscular mycorrhiza symbiosis. Int J Mol Sci. 2018; 19: 3146. https://doi.org/10.3390/ijms19103146 PMID: 30322086

38. Pons S, Fournier S, Bercovici C, Bécard G, Rochange S, Frei D, Frey N, et al. Phytohormone production by the arbuscular mycorrhizal fungus *Rhizophagus irregularis*. PLoS One. 2020; 15: e0240886.

39. Torelli A, Trotta A, Acerbi L, Arcidiacono G, Berta G, Branca C. IAA and ZR content in leek (*Allium porrum* L.), as influenced by P nutrition and arbuscular mycorrhizae, in relation to plant development. Plant and Soil. 2000; 226: 29–35.

40. Shaul-Keinan O, Gadkar V, Ginzberg I, Grünzweig JM, Chet I, Elad Y, et al. Hormone concentrations in tobacco roots change during arbuscular mycorrhizal colonization with *Glomus intraradices*. New Phytol. 2002; 154: 501–507.

41. Fitze D, Wiepning A, Kaldorf M, Ludwig-Müller J. Auxins in the development of an arbuscular mycorrhizal symbiosis in maize. J Plant Physiol. 2005; 162: 1210–1219. https://doi.org/10.1016/j.jplph.2005.01.014 PMID: 16323272

42. Meixner C, Ludwig-Müller J, Miersch O, Gresshoff P, Staehelin C, Vierheilig H. Lack of mycorrhizal autoregulation and phytohormonal changes in the supernodulating soybean mutant nts1007. Planta. 2005; 222: 709–715. https://doi.org/10.1007/s00425-005-0003-4 PMID: 16025340

43. Ludwig-Müller J, Güther M. Auxins as signals in arbuscular mycorrhiza formation. Plant Signal Behav. 2007; 2: 194–196. https://doi.org/10.4161/psb.2.3.4152 PMID: 19704695

44. Buendia L, Ribeyre C, Bensmihen S, Lefebvre B. *Brachypodium distachyon tar2l* hypo mutant shows reduced root developmental response to symbiotic signal but increased arbuscular mycorrhiza. Plant Signaling & Behavior. 2019; 14: e1651608.

45. Etemadi M, Gutjahr C, Couzigou JM, Zouine M, Lauressergues D, Timmers A. Auxin perception is required for arbuscule development in arbuscular mycorrhizal symbioses. Plant Physiol. 2014; 166: 281–292. https://doi.org/10.1104/pp.114.246595 PMID: 25096975

46. Kohlen W, Ng JLP, Deinum EE, Mathesius U. Auxin transport, metabolism, and signaling during nodule initiation: Indeterminate and determinate nodules. J Exp Bot. 2018; 69: 229–244. https://doi.org/10.1093/jxb/erx308 PMID: 28992078

47. Garraway MO. Stimulation of *Armillaria mellea* growth by plant hormones in relation to the concentration and type of carbohydrate, Eur J For Path. 1975; 5: 35–43.

48. Yan QH, Liu DY, Wang CT. Effect of NAA on the growth of *Armillaria mellea* mycelium and on the activities of catalase and superoxide dismutase. Journal of microbiology. 2000; 20: 19–22. (Chinese)

49. Cao WQ, Xu JT. Liquid culture of the fruiting body of *Armillaria mellea*. CHIN MED MAT. 1992; 15: 3–4. (Chinese)

50. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA Seq data with or without a reference genome. BMC Bioinformatics. 2011; 12: 323. https://doi.org/10.1186/1471-2105-12-323 PMID: 21816040

51. Xie C, Mao X, Huang J, Ding Y, Wu J, Dong S, et al. KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. Nucleic Acids Res. 2011; 39: W316–W322. https://doi.org/10.1093/nar/gkr483 PMID: 21715388

52. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods: A Companion to Methods in Enzymology. 2001; 25: 402–408. https://doi.org/10.1006/meth.2001.1292 PMID: 11846909

53. Voges MJEE, Bai Y, Schulze-Lefert P, Sattely ES. Plant-derived coumarins shape the composition of an Arabidopsis synthetic root microbiome. Proc Natl Acad Sci USA. 2019; 116: 12558–12565.

54. Harbort CJ, Hashimoto M, Inoue H, Niu Y, Guan R, Rombola AD, et al. Root-secreted coumarins and the microbiota interact to improve iron nutrition in *Arabidopsis*. Cell Host Microbe. 2020; 28: 825–837.

55. Hilbert M, Voll LM, Ding Y, Hofmann J, Sharma M, Zuccaro A. Indole derivative production by the root endophyte *Piriformospora indica* is not required for growth promotion but for biotrophic colonization of barley roots. New Phytol. 2012; 196: 520–534.
56. Bacteria Ludwig-Müller J. and fungi controlling plant growth by manipulating auxin: balance between development and defense. J Plant Physiol. 2015; 172: 4–12.

57. Kwasna H and Lakomy P. Stimulation of Armillaria ostoyae vegetative growth by tryptophol and rhizomorph produced by Zygorhynchos moelleri. Eur J For Path. 1998; 28: 53–61.

58. Gifford ML, Dean A, Gutierrez RA, Coruzzi GM, Birnbaum KD. Cell-specific nitrogen responses mediate developmental plasticity. Proc Natl Acad Sci USA. 2008; 105: 803–808. https://doi.org/10.1073/pnas.0709559105 PMID: 18180456

59. Vidal EA, Araus V, Lu C, Parry G, Green PJ, Coruzzi GM, et al. Nitrate-responsive miR393/AFB3 regulatory module controls root system architecture in Arabidopsis thaliana. Proc Natl Acad Sci USA. 2010; 107: 4477–4482.

60. Krouk G, Lacombe B, Bielach A, Perrine-Walker F, Malinska K, Mounier E, et al. Nitrate-regulated auxin transport by NRT1.1 defines a mechanism for nutrient sensing in plants. Dev Cell. 2010; 18: 927–937. https://doi.org/10.1016/j.devcel.2010.05.008 PMID: 20627075

61. Ma W, Li J, Qu B, He X, Zhao X, Li B, et al. Auxin biosynthetic gene TAR2 is involved in low nitrogen-mediated reprogramming of root architecture in Arabidopsis. Plant J. 2014; 78: 70–79.

62. Zhang S, Zhu L, Shen C, Ji Z, Zhang H, Zhang T, et al. Natural allelic variation in a modulator of auxin homeostasis improves grain yield and nitrogen use efficiency in rice. Plant Cell. 2021; 33: 566–580. https://doi.org/10.1093/plcell/koaa037 PMID: 33955496

63. Pérez-dolos Santos FJ, Riego-Ruiz L. Gin3 is a main regulator of nitrogen assimilation in Candida glabrata. Microbiology. 2016; 162: 1490–1499.

64. Hou R, Jiang C, Zheng Q, Wang C, Xu JR. The AreA transcription factor mediates the regulation of deoxynivalenol (DON) synthesis by ammonium and cyclic adenosine monophosphate (cAMP) signaling in Fusarium graminearum. Molecular plant pathology. 2015; 16: 987–999.