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# Auxin, Cytokinin, and Ethylene Involved in Rice N Availability Improvement Caused by Endophyte *Phomopsis liquidambari*

**Xin Li[1] · Jun Zhou[1] · Ri-Sheng Xu[1] · Mei-Yao Meng[1] · Xiao Yu[1] · Chuan-Chao Dai[1]**

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**Abstract** Nitrogen (N) is one of the most limiting nutrients for rice yield. There is mounting evidence that the endophytic fungus *Phomopsis liquidambari* B3 can establish a mutualistic symbiotic relationship with rice, enhancing N uptake and metabolism in rice (*Oryza sativa* L.). To examine the mechanism underlying the effect of B3 on nitrogen accumulation and metabolism in rice plants, a pot experiment was conducted to examine the N and phytohormone levels in response to endophyte infection at four whole growth durations during exposure to different N levels. Our results showed that the contents of auxin, cytokinin, and ethylene in rice were significantly enhanced by B3 under low N levels at different growth durations; B3 symbiosis increased N accumulation and rice yield and induced the expression of some genes related to N uptake and metabolism. To further verify that B3 symbiosis enhances N use in rice by regulating phytohormones, we performed a hydroponic experiment in which exogenous phytohormones and their specific inhibitors were applied. The results showed that the application of exogenous auxin, cytokinin, and ethylene increased the rice content of nitrogen, and their inhibitors decreased the amount of nitrogen absorbed in rice. As expected, B3 infection alleviated the negative effect caused by inhibitors slightly. In summary, we conclude that *P. liquidambari* symbiosis may regulate the content of auxin, cytokinin, and ethylene to improve N use in rice.

**Keywords** *Phomopsis liquidambari* · Rice · Nitrogen uptake · Nitrogen metabolism · Phytohormones · Gene expression

## Introduction

N, which is considered a fundamental element of many cell components, such as proteins and nucleotides, is a limiting factor for plant growth and development. So a large amount of N fertilizers are applied to increase crop yields (Wang and others 2012; Mu and others 2016). Rice (*Oryza sativa* L.) is the staple food of approximately 3.5 billion of the world's population. Foley and others (2011) and Mueller and others (2012) suggested that by 2050, the global population will increase by 2–3 billion, implying that demands for agricultural land and N fertilizers are likely to grow substantially. Unfortunately, overuse of N fertilizer has resulted in the pollution of oceans and rivers, eutrophication, and wasting of resources (Wang and others 2012; Zhang and others 2015). Increasing NUE (nitrogen utilization efficiency) and reducing the application of N fertilizer in crop plants have to be settled urgently (Wang and others 2012; Yousaf and others 2016). However, measures to improve crop NUE have limitations, such as a long cycle, high cost, high technological demands, and difficulties related to yield and disease resistance, and therefore an increasing number of researchers hope to take the road of ecological intensification through biological processes, using the symbiotic relationship between plants and microbes to solve these problems.

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✉ Chuan-Chao Dai
daichuanchao@njnu.edu.cn

[1] Jiangsu Key Laboratory for Microbes and Functional Genomics, Jiangsu Engineering and Technology Research Center for Industrialization of Microbial Resources, College of Life Sciences, Nanjing Normal University, Jiangsu, China

Recently, the potential for microbes to enhance nutrient availability and improve crop growth has captured the attention of researchers, and an increasing reliance on biological processes and plant–microbe interactions may be one of the most promising measures to cope with these problems (Tikhonovich and others 2011). Endophytes play an irreplaceable role in plant N nutrition. The formation of arbuscular mycorrhiza (AM) has been found to induce plant N transporter genes (Koegel and others 2013) and to improve the ability of plants to get nitrogen from both organic and inorganic sources (Tian and others 2010). AM infection in the soybean/maize intercropping system increased N fixation efficiency in soybean (Meng and others 2015). N metabolism is also facilitated by the endophytic fungus, *Piriformospora indica*, which improves the growth of tobacco roots by stimulating the expression of related enzymes such as starch-degrading enzyme and nitrate reductase (Sherameti and others 2005). In contrast, ascomycete endophytes have frequently been found to improve host plant growth by improving the capacity of plants to capture N from the soil. A previous study found that *Phomopsis liquidambari*, a broad host endophyte fungi, can develop a symbiotic relationship with rice (Zhou and others 2017; Yang and others 2013), improve uptake and metabolism of N in rice, promote the growth and yield of rice, and substantially reduce the required amount of soil N fertilizer (Yang and others 2014), and produce abscisic acid and 3-indole acetic acid in vitro (Chen and others 2011). Moreover, previous work has shown that *P. liquidambari* increased the relative transcript levels of genes involved in N use in rice at the seedling stage (Yang and others 2013). The above results indicate the useful impacts of *P. liquidambari* on nitrogen uptake and metabolism in rice; however, very little is known about the underlying mechanisms.

Diverse phytohormones are involved in the acquisition of mineral nutrition by plants. The effects of these phytohormones on N assimilation and gene acquisition have been demonstrated, revealing a positive retro-control of growth on nutrient uptake and assimilation, which is very likely to be supported by dedicated signaling pathways (Krouk and others 2011). Under natural conditions, most nutrients are absorbed into plants via the roots. On the one hand, plant hormones affect the absorption of nutrients by regulating root development. For example, the absence of the ethylene signal transduction genes, *ETR1* and *EIN2*, and the application of the ethylene biosynthesis inhibitor of AVG resulted in the inhibition of lateral root growth when the entire root system was supplied with high nitrogen levels. These results indicate that ethylene is involved in the modulation of N in lateral roots (Tian and others 2009). On the other hand, the hormones can directly affect nutrient absorption and utilization by root cells. For example, increased transport of cytokinins from underground parts to aboveground parts appears to induce response regulator genes that are involved in N signal transduction and to increase nitrate reductase expression in leaves.

Increasing evidence shows that endophytic fungi can produce different plant hormones to enhance the growth of their host plants (Waqas and others 2012; Cassán and others 2013) or regulate the production of hormones (ethylene, auxin, and cytokinin) in host plants. Research by Sirrenberg indicated that *P. indica* could produce IAA to improve the growth of plants (Sirrenberg and others 2007). The endophytes *Puccinia glomerata* and *Penicillium* sp. have been reported to secrete activated GAs (GA1, GA3, GA4, and GA7) and IAA (Waqas and others 2012). Our previous research indicated that a fungal endophyte, *P. liquidambari*, can produce 6213.6 pmol $L^{-1}$ 3-indole acetic acid and 25,117 pmol $L^{-1}$ abscisic acid in vitro (Chen and others 2011). Based on the above results, we hypothesize that the endophytic fungus *P. liquidambari* can enhance the level of N metabolism and uptake in rice by regulating the content of phytohormones. To test this hypothesis, we performed pot and hydroponic experiments under different N levels.

## Materials and Methods

### Fungal Strain, Plant Seeds, and Paddy Soil

*Phomopsis liquidambari*, which was isolated from *Bischofia polycarpa*, was preserved on potato dextrose agar (PDA) at 4 °C.

The rice cultivar used herein was "Wuyunjing 23" (a common cultivar in China), a japonica subspecies of *O. sativa* L. The experimental soil was collected from the experimental rice fields in Nanjing Normal University, and then was air-dried, sieved, and added to experimental pots (26 cm in diameter, 34 cm in height). The soil characteristics are described in the Supplementary Material.

### Endophytic Fungal Infection and Cultivation of Rice Seedlings

First, *P. liquidambari* was activated in potato dextrose broth (PDB) at 28 °C at 160 rpm for 3 days, and then 4% seed culture broth was transferred to the new PDB at 28 °C at 160 rpm for 4 days. In total, 3.21 g (0.345 g dry weight) of fungal mycelia was collected and diluted with 250 mL sterile distilled water (SDW).

The dehulled rice seeds were sterilized in 75% ethanol for 10 min, and then dipped in 6% NaClO for 15 min. The sterilized seeds were divided into two parts and then placed on culture dishes (15 cm dia., 80 grains per dish). The above mentioned fungal suspension (80 mL per dish) was

added to the endophyte-infected group (E+). For the uninfected group (E−), 80 mL of SDW was added as a control (Yang and others 2013, 2014). The seeds were germinated in the dark for 48 h and then grown in an incubator for 4 days under conditions of 29/25 °C day/night with a 16/8-h photoperiod and a light intensity of 250 μmol $m^{-2}$ $s^{-1}$.

### Pot Experimental Design and Plant Growth Conditions

Germinated rice seeds were transplanted into pots (35 cm in height and 25 cm in diameter) containing 15 kg of paddy soil. After 20 days of growth (mid-June 2015), seedlings at similar developmental stages were selected and transplanted into pots accommodating seedlings per pot and grown under experimental field conditions.

Pot experiments were arranged in a 3 × 2 factorial design, with the level of supplied N and endophyte infection as the main factors. The N treatments consisted of three gradients: low nitrogen (LN), 1.25 g N per pot; medium nitrogen (MN), 2.5 g N per pot; and high nitrogen (HN), 3.75 g N per pot. The method used for N, P, and K fertilizer application is described in the Supplementary Material.

### Hydroponic Experimental Design and Plant Growth Conditions

The rice seeds were sterilized as mentioned previously and then were placed on culture dishes containing 80 mL of sterilized deionized water (15 cm dia., 80 grains per dish). The seeds were germinated in the dark for 48 h and then grown in an incubator for 4 days under conditions of 29/25 °C day/night with a 16/8-h photoperiod and a light intensity of 250 μmol $m^{-2}$ $s^{-1}$. The seeds were then placed in planting baskets and cultivated hydroponically in a 150-mL triangle bottle containing 125 mL of IRRI (International Rice Research Institute) nutrient solution at 1/4th strength with some adjustment (Yoshida and others 1976). The nutrient solution contained 1.0 mM $NH_4NO_3$, 0.32 mM $NaH_2PO_4$, 1.0 mM $K_2SO_4$, 1.0 mM $CaCl_2$, 1.7 mM $MgSO_4$, 0.072 mM Fe-EDTA, 0.2 mM $Na_2SiO_3$, 9.1 mM $MnCl_2$, 0.5 mM $ZnSO_4$, 0.5 mM $CuSO_4$, 18.0 mM $H_3BO_3$, and 0.526 mM $H_2MoO_4$, with pH adjusted to 5.5. The root zones were inhibited by wrapping the triangle bottles with aluminum foil, and the rice seedlings were grown in a growth cabinet for 18 days. The nutrient solution was changed every 2 days. Phytohormones, specific inhibitors, and endophytes were applied to the 10-day-old rice seedlings: auxin (IAA, 10 μM), cytokinin (CTK, 1 μM), 1-aminocyclopropanecarboxylic acid (ACC, 100 μM), 2-(4-chlorophenoxy) isobutyric (PCIB, 10, 50, 100 μM), O-(carboxymethyl) hydroxylamine hemihydrochloride (AOA, 10, 50, 100 μM), and endophyte fungal suspension. All reagents were purchased from Sigma-Aldrich company (St. Louis, MO, USA).

The above solutions were dissolved in double-distilled water or 96% ethyl alcohol and then sterilized by filtration through a 0.22-μm sterile filter. In cases of concomitant treatment with endophyte, inhibitor, and plant hormones, the inhibitor was applied 24 h before endophyte infection (Zhou and others 2016).

### Sample Collection and Preparation

To analyze the physiological and biochemical indexes, plant samples from the pot experiment were collected at 9:00 am for the four rice growing stages: seedling stage (S1), tillering stage (S2), heading stage (S3), and ripening stage (S4). For the hydroponic experiment, 24-day-old rice seedlings were collected. Five plants collected for each treatment randomly were used in the analysis. Parts of the plant samples were dried for analysis of total N and biomass, and the others were immediately frozen in separate containers in liquid nitrogen and stored at −80 °C.

At every growth stage, rice shoots and roots were separated, washed, and then placed in an oven at 105 °C for half an hour to inactivate the enzymes. Finally, they were dried at −80 °C to a constant weight. After recording the dry weight (DW), the dried samples were milled, passed through a 1-mm screen, and stored for chemical analysis. All plants in pots were used to measure the grain yield after harvesting.

### Analysis of Nitrate Reductase (NR) Activity

To assay NR activity, 0.5 g fresh tissue was cut into pieces and ground in 4 mL extraction buffer on ice. The compound was first centrifuged at 5000 rpm for 10 min and then incubated at 25 °C. After 30 min, 1 mL 1% (w/v) sulfanilamide was added to terminate the reaction, and then 2 mL 0.01% (w/v) *N*-naphthyl-(1)-dihydrochloride was added followed by incubation at 25 °C for 15 min for color development. Finally the absorbance was measured at 540 nm (Yang and others 2013, 2014).

### Analysis of Glutamine Synthetase (GS) Activity

To determine the total GS activities, fresh plant tissues were cut into pieces and ground in extraction buffer on ice. The color reaction was conducted according to the method reported by Husted and others (2002): first, the homogenates were centrifuged at 10,000 rpm at 4 °C for 25 min, then the supernatant was removed. Assay buffer was used to measure the total GS activity at 37 °C. After 30 min, acidic $FeCl_3$ solution was added to terminate the reaction. After 10 min of color development, the mixture was centrifuged

at 4000 rpm for 10 min, and the 'absorbance' of the supernatant was measured at 540 nm (Husted and others 2002; Yang and others 2014).

### Determination of Free $NO_3^-$, Free $NH_4^+$, and Total N in Rice Plants

To assay the contents of free $NO_3^-$ and $NH_4^+$, fresh plant tissues were ground in cold extraction buffer, and the homogenates were centrifuged at 12,000×*g* at 4 °C for 20 min (Oliveira and others 2002). The content of free $NO_3^-$ was determined according to the method reported by Eckhardt and others (1999), and the absorbance was determined at 540 nm. The content of free $NH_4^+$ was measured according to Gordon and others (1978), and the absorbance at 480 nm was determined. A Kjeltec™ 2100 semi-automatic analyzer was used to determine the total N (Yang and others 2013, 2014). The nitrogen harvest index (NHI) was defined as the ratio of total N in the grain to the total N in the plant. The nitrogen use efficiency (NUE) was defined as the grain yield per unit of N available from the soil, including N fertilizer (Yang and others 2013, 2014).

### Quantitative Real-time PCR Analysis of Rice Genes

Total RNA of rice was extracted with TRIzol (Invitrogen, USA). Reverse transcription was conducted with cDNA Synthesis Kit (Invitrogen), and then real-time PCR was conducted with gene-specific primers, which are shown in Table S1. The reactive step of qPCR followed: stage 1, 95 °C for 5 min; stage 2, 40 cycles of 10 s at 95 °C, 30 s at 60 °C; stage 3, 15 s at 95 °C, 60 s at 60 °C, and 15 s at 95 °C. Amplification of the target gene was monitored every cycle with SYBR Green (Yang and others 2013, 2014). The relative expression of the target genes was calculated using the log2 method (Kiba and others 2011).

### Measurement of Phytohormones

Auxin (IAA) was extracted according to the following method: 2 g of shoots or roots were ground in liquid nitrogen with 50 mg BHT, 50 mg PVP, and 10 mL methanol overnight. The mixture was centrifuged at 10,000 rpm for 15 min (Zhou and others 2016). A Plant IAA Assay Kit was used to determine the IAA content in the supernatant (Zhou and others 2016).

Gibberellin (GA) was extracted from the plants according to Zhou and others (2016). One gram of rice was milled with phosphate-buffered saline (pH 7.4); then the mixture was centrifuged at 4000 rpm for 15 min. A Plant GA Assay Kit was used to assay the GA content in the supernatant (Zhou and others 2016).

Ethylene (ETH) was extracted according to Yuan and others (2016). One gram of rice was milled in liquid nitrogen with 5 mL phosphate-buffered saline (pH 7.5); then the mixture was centrifuged at 3000×*g* for 15 min. A Plant ETH Assay Kit was used to determine the content of ETH in the supernatant (Yuan and others 2016).

Brassinolide (BL) was extracted according to Swaczynova and others (2007) with some modifications. Two grams of fresh rice tissues was ground in liquid nitrogen and extracted twice in 10 mL ice-cold 80% (v/v) methanol in an ultrasonic bath for 30 min. The mixture was centrifuged at 12,000×*g* at 4 °C for 10 min, and the supernatant was evaporated to dryness, dissolved in 1 mL methanol, and stored at −20 °C for analysis. A Plant BL Assay Kit was used to measure the BL content.

Cytokinin (CTK) was extracted using the following method: 2 g rice plant tissue were ground in liquid nitrogen with 40 ppm sodium diethyldithiocarbamate and 2% (w/w) PVP, and then 16 mL ice-cold methanol was used for extraction at 4 °C overnight. Next, the mixture was centrifuged at 4000×*g* for 15 min, the supernatant was evaporated. Ethylacetate was added to the residue. Finally, the mixture was evaporated to dryness and dissolved in 300 µL 95% ethyl alcohol. A Plant CTK Assay Kit was used to measure the CTK content (Zhou and others 2016).

Abscisic acid (ABA) was extracted and measured using the following method (Wang and others 2015; Zhou and others 2016): Two grams of rice tissue were milled with 15 mL 80% methanol (v/v) overnight. The mixture was centrifuged at 8000×*g* for 15 min, and the supernatant was evaporated. Finally, the mixture was evaporated, and dissolved in 300 µL of high performance liquid chromatography (HPLC) mobile phase (acetonitrile: 1.8% acetic acid, 1:1, v/v).

The content of ABA was measured by HPLC. The flow rate was 0.5 mL $min^{-1}$, and the column was maintained at 25 °C with detection at 260 nm (Wang and others 2015; Zhou and others 2016).

All plant hormone assay kits were purchased from Fankel Biological Technology company (Shanghai, China). The plant hormone assay kit contains 30-fold concentrated wash solution, ELISA reagent, microplate, sample diluent, reagent A, reagent B, and stop solution. The steps are as follows: Firstly, add the sample. The blank holes (without sample and ELISA reagents) and the sample holes were set respectively, 40 µL of sample diluent was added into blank holes and sample holes, then 10 µL sample was added into sample holes. The plate was incubated at 37 °C for 30 min. Secondly, washing. A 30-fold concentrated detergent was diluted 30-fold with distilled water. Discard the liquid in the plate, dry it. Fill the wash solution per well for 30 s, then discard it. Thirdly, 50 µL ELISA reagent was added to sample holes. The plate was incubated at 37 °C for 30 min

and washed. Reagent A (50 µL) was added then 50 µL of reagent B was added. The plate was incubated at 37 °C for 10 min. Then 50 µL stop solution was added to stop the reaction. Lastly, the OD at 450 nm was determined.

### Statistical Analysis

All experiments were performed in triplicate with three biological replicates in each repeat. All statistical analyses, including the means and standard error (SE), were calculated using SPSS Statistics 18.0 (SPSS, Chicago, IL, USA). All data represent an average of three biological replicates. When an analysis only consisted of a control and an experimental group, the experiments were analyzed by one-way ANOVA using SPSS Statistics 18.0 software; when an analysis consisted of the three levels of nitrogen and two levels of endophytes, the experiments were analyzed by two-way ANOVA using SPSS Statistics 18.0 software. The data were considered significantly different at $P < 0.05$.

## Results

### Effects of *P. liquidambari* on Rice Biomass and Yield

To determine the beneficial effects of endophyte infection on rice plants, we compared the plant biomass and yield index *of P. liquidambari*-infected rice with uninfected plants in the presence of different levels of N. Table 1 displays the growth dynamics of rice at different growth stages. As shown in Table 1, at each growth stage, the biomasses of both rice shoots and roots were significantly increased in E+ treatments under LN. Compared with the E− treatment, under LN, the total biomasses of rice in E+ treatments increased by 24.16, 12.72, 19.71, and 9.51% at the four growth stages, respectively. Under middle and high nitrogen levels, however, *P. liquidambari* infection did not induce significant changes. Moreover, the rice of E+ treatments displayed grain yield increases of 12.26% under LN (Table 1).

**Table 1** Effect of B3 infection on rice growth and N contents at four growth stages

| Growth stage | N level | Endophyte status | Shoot | | Root | | Seed | | N accumulation (mg plant$^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| | | | Biomass (g plant$^{-1}$) | N content (mg g$^{-1}$) | Biomass (g plant$^{-1}$) | N content (mg g$^{-1}$) | Biomass (g plant$^{-1}$) | N content (mg g$^{-1}$) | |
| S1 | LN | E− | 1.53 ± 0.047 | 25.19 ± 0.31 | 0.25 ± 0.02 | 17.12 ± 0.33 | – | – | 42.25 ± 2.68 |
| | | E+ | 1.89 ± 0.051** | 26.76 ± 0.26** | 0.32 ± 0.04** | 18.28 ± 0.27** | – | – | 55.76 ± 3.51** |
| | MN | E− | 2.09 ± 0.13 | 27.18 ± 0.22 | 0.37 ± 0.03 | 19.54 ± 0.17 | – | – | 64.16 ± 5.01 |
| | | E+ | 2.22 ± 0.16* | 27.44 ± 0.16 | 0.42 ± 0.02* | 19.83 ± 0.27 | – | – | 68.91 ± 4.03 |
| | HN | E− | 2.52 ± 0.15 | 28.13 ± 0.17 | 0.47 ± 0.02 | 20.71 ± 0.28 | – | – | 82.03 ± 5.89 |
| | | E+ | 2.50 ± 0.18 | 29.06 ± 0.18 | 0.48 ± 0.03 | 20.97 ± 0.24 | – | – | 83.27 ± 6.37 |
| S2 | LN | E− | 5.74 ± 0.11 | 27.84 ± 0.14 | 1.02 ± 0.09 | 25.09 ± 0.15 | – | – | 183.02 ± 6.89 |
| | | E+ | 6.38 ± 0.21** | 29.82 ± 0.11** | 1.24 ± 0.04** | 26.81 ± 0.08** | – | – | 220.21 ± 7.58** |
| | MN | E− | 6.79 ± 0.15 | 30.51 ± 0.19 | 1.49 ± 0.07 | 27.73 ± 0.27 | – | – | 243.29 ± 8.06 |
| | | E+ | 6.80 ± 0.30 | 30.78 ± 0.26 | 1.56 ± 0.08 | 27.81 ± 0.26 | – | – | 250.98 ± 5.01 |
| | HN | E− | 7.45 ± 0.26 | 31.64 ± 0.22 | 1.65 ± 008 | 28.98 ± 0.16 | – | – | 282.62 ± 6.42 |
| | | E+ | 7.33 ± 0.18 | 31.78 ± 0.29 | 1.78 ± 1.03 | 29.13 ± 0.29 | – | – | 280.90 ± 4.90 |
| S3 | LN | E− | 16.25 ± 1.17 | 20.53 ± 0.15 | 4.30 ± 0.07 | 11.43 ± 0.22 | – | – | 383.25 ± 21.78 |
| | | E+ | 19.78 ± 1.44** | 21.20 ± 0.21** | 4.82 ± 0.21** | 12.73 ± 0.23** | – | – | 460.03 ± 18.16** |
| | MN | E− | 20.78 ± 1.24 | 21.7 ± 0.27 | 5.52 ± 0.15 | 14.65 ± 0.28 | – | – | 530.88 ± 21.08 |
| | | E+ | 21.17 ± 1.71 | 21.99 ± 0.28 | 5.60 ± 0.20 | 14.86 ± 0.25 | – | – | 535.76 ± 19.49 |
| | HN | E− | 26.11 ± 0.96 | 23.67 ± 0.09 | 5.84 ± 0.19 | 15.50 ± 0.21 | – | – | 688.69 ± 18.76 |
| | | E+ | 26.83 ± 1.15 | 23.81 ± 0.16 | 5.95 ± 0.22 | 15.72 ± 0.25 | – | – | 706.10 ± 22.13 |
| S4 | LN | E− | 23.36 ± 1.6 | 7.26 ± 0.28 | 5.90 ± 0.25 | 7.05 ± 0.10 | 22.45 ± 0.96 | 11.42 ± 0.19 | 470.82 ± 24.31 |
| | | E+ | 25.24 ± 0.98 | 7.93 ± 0.32** | 6.05 ± 0.16 | 7.14 ± 0.12 | 25.34 ± 0.55** | 12.8 ± 0.11** | 560.09 ± 20.02** |
| | MN | E− | 26.40 ± 1.3 | 10.09 ± 0.16 | 6.80 ± 0.21 | 7.73 ± 0.14 | 25.80 ± 1.06 | 13.57 ± 0.16 | 670.31 ± 17.57 |
| | | E+ | 27.22 ± 0.87 | 10.01 ± 0.26 | 6.91 ± 0.14 | 7.79 ± 0.08 | 26.36 ± 0.88 | 13.77 ± 0.27 | 689.95 ± 26.78 |
| | HN | E− | 28.03 ± 1.3 | 11.37 ± 0.33 | 7.37 ± 0.23 | 8.24 ± 0.09 | 27.31 ± 0.62 | 14.04 ± 0.12 | 740.17 ± 19.65 |
| | | E+ | 28.39 ± 0.75 | 11.54 ± 0.25 | 7.22 ± 0.14 | 8.41 ± 0.10 | 27.75 ± 0.78 | 14.08 ± 0.09 | 769.42 ± 25.90 |

Values are means +/− standard errors for three biological replicates. Asterisks indicate significant differences (*$P < 0.05$; **$P < 0.01$). *LN* low nitrogen, *MN* middle nitrogen, *HN* high nitrogen, *E+* endophyte-infected, *E−* endophyte-uninfected

### Effects of *P. liquidambari* on N Content

To determine whether *P. liquidambari* infection induced changes in rice nitrogen accumulation, we examined the contents of total nitrogen in rice. As shown in Table 1, at the S1, S2, and S3 stages, *P. liquidambari* infection caused a substantial increase in total N of rice under the low N level. The content of total N in shoots and roots of infected rice was enhanced by 6.23 and 6.78% at the S1 stage under LN; by 7.11 and 6.86% at the S2 stage; and by 3.26 and 11.37% at the S3 stage, respectively. Additionally, under LN, the N contents of the grain with E+ treatment increased by 12.08% at the S4 stage. Under middle and high N levels, *P. liquidambari* infection did not induce significant changes.

Total N accumulation in *P. liquidambari*-infected rice was also markedly enhanced under the low nitrogen level. Under LN, compared with the E− treatments, total N accumulation with E+ treatment increased by 31.98, 20.32, 20.04, and 18.96% in the four growth stages. In addition, under the low nitrogen level, *P. liquidambari* infection lead to a mean increase in the NHI and NUE of rice by 6.53 and 12.63%, respectively (Table 2).

### Effects of *P. liquidambari* on the Activities of NR and GS in Rice

To determine the relationship between effects caused by *P. liquidambari* infection on rice and the key enzymes involved in N metabolism in rice, we examined the activities of NR and GS in host rice. The results showed that inoculation of *P. liquidambari* induced a mean increase in the activities of NR and GS when supplied with low nitrogen (Figs. 1, 2). At the S1 stage, compared with uninfected rice, NR activity in endophyte-infected rice shoots and roots increased by 13.67 and 16.67% under LN and by 6.44 and 16.65% under MN, respectively. At the S1 stage, compared with uninfected rice, GS activities in shoots and roots increased by 44.59 and 39.02% under LN and by 7.69 and 11.26% under MN, respectively. At the S2 stage, compared with uninfected plants, the activity of NR of the infected rice shoots and roots increased by 11.71 and 10.08% under LN, respectively. In contrast, the activity of GS in rice

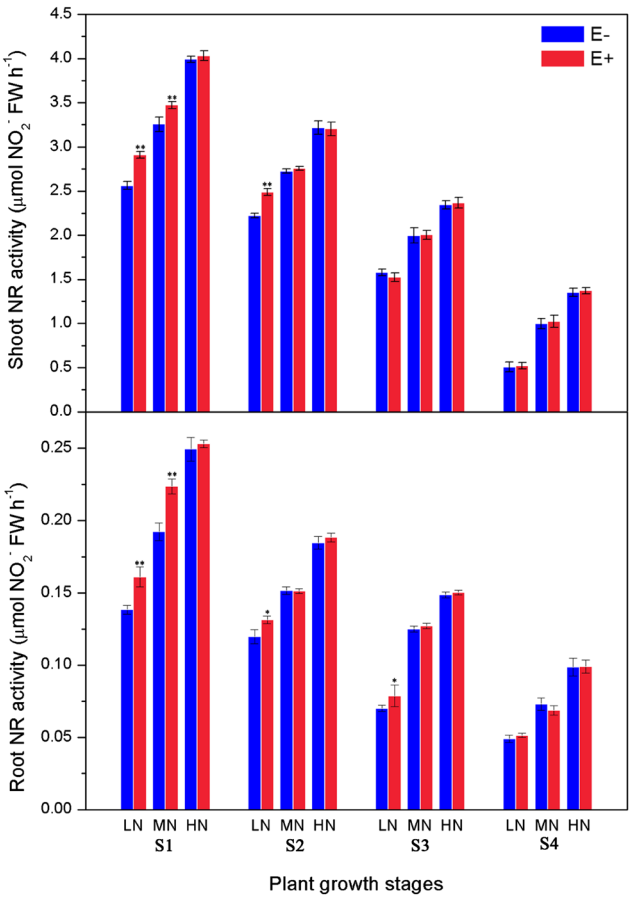


**Fig. 1** Nitrate reductase (NR) activities in rice with endophyte treatment (E+) and control treatment (E−). Values are means for three biological replicates. *Bars* denote the standard error of the mean. *Asterisks* indicate significant differences (*$P<0.05$; **$P<0.01$). *LN* low nitrogen, *MN* middle nitrogen, *HN* high nitrogen, *E+* endophyte-infected, *E−* endophyte-uninfected. (Color figure online)

**Table 2** Effect of B3 infection on yield parameters, NHI and NUE in rice exposed to different N levels

| N level | Endophyte status | Panicles per plant (n) | Tiller number plant (n) | Grains per panicle (n) | Seeds set (%) | 1000-grain weight (g) | Nitrogen harvest index (%) | Nitrogen use efficiency (g $g^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| LN | E− | 6.36 ± 0.31 | 6.27 ± 0.22 | 120.97 ± 1.42 | 85.21 ± 0.79 | 26.67 ± 0.91 | 54.49 ± 0.76 | 24.55 ± 0.28 |
| | E+ | 7.14 ± 0.26* | 7.24 ± 0.30** | 132.26 ± 0.98** | 88.40 ± 1.42** | 29.56 ± 0.43** | 58.05 ± 0.48** | 27.65 ± 0.31** |
| MN | E− | 7.74 ± 0.49 | 7.89 ± 0.29 | 143.50 ± 1.22 | 89.13 ± 1.42 | 31.11 ± 0.62 | 53.36 ± 0.54 | 19.43 ± 0.27 |
| | E+ | 7.86 ± 0.45 | 7.96 ± 0.44 | 146.12 ± 0.76* | 91.03 ± 0.73* | 31.56 ± 0.70 | 54.94 ± 0.39 | 19.60 ± 0.25 |
| HN | E− | 8.67 ± 0.52 | 8.50 ± 0.52 | 153.06 ± 1.5 | 91.53 ± 0.56 | 32.27 ± 0.46 | 50.05 ± 0.51 | 15.66 ± 0.21 |
| | E+ | 8.93 ± 0.40 | 8.95 ± 0.33 | 154.18 ± 1.42 | 91.80 ± 0.75 | 32.34 ± 0.84 | 50.13 ± 0.42 | 15.83 ± 0.28 |

Values are means for three biological replicates. Asterisks indicate significant differences (*$P<0.05$; **$P<0.01$). *LN* low nitrogen, *MN* middle nitrogen, *HN* high nitrogen, *E+* endophyte-infected, *E−* endophyte-uninfected

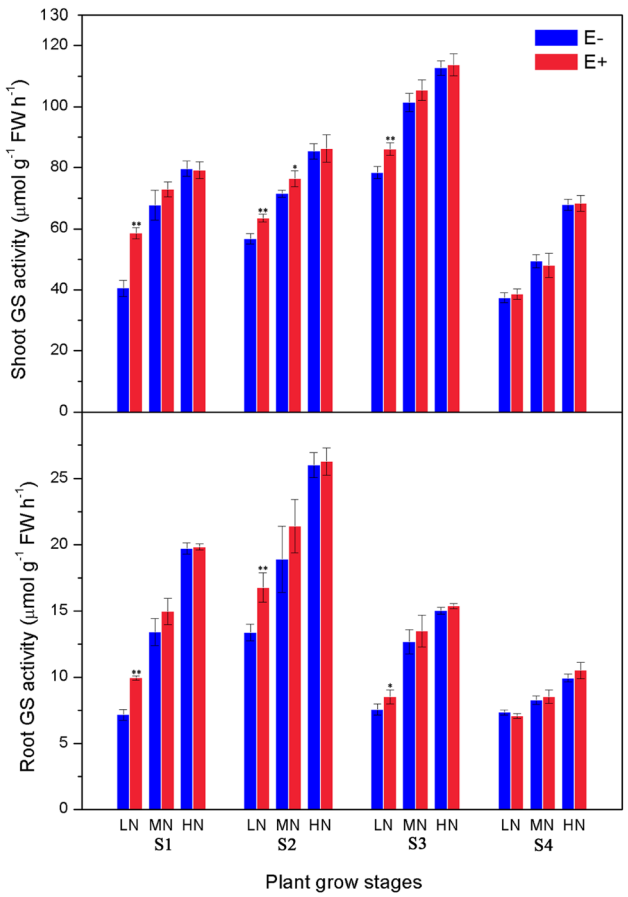


**Fig. 2** Glutamine synthetase (GS) activities in rice with endophyte treatment (E+) and control treatment (E−). Values are means for three biological replicates. *Bars* denote the standard error of the mean. *Asterisks* indicate significant differences (*$P<0.05$; **$P<0.01$). *LN* low nitrogen, *MN* middle nitrogen, *HN* high nitrogen, *E+* endophyte-infected, *E−* endophyte-uninfected. (Color figure online)

shoots and roots increased by 11.99 and 25.36% under LN, and the activity of GS in rice shoots increased by 6.90% under MN. At the S3 stage, *P. liquidambari* infection resulted in a mean increase in NR activity of 12.86% in rice roots under LN, and an increase in GS activity by 9.81% in rice shoots under LN. There were no apparent differences under HN during the four rice growth stages.

### Expression Levels of Genes Related in N Uptake

The expression levels of genes involved in nitrogen uptake in rice were examined to study the potential mechanisms of changes caused by *P. liquidambari* on nitrogen uptake in rice. With increasing N levels or growing stage, these differences diminished or disappeared. As shown in Table 3, at the S1 stage, fungal infection strongly up-regulated the transcript levels of *OsNRT2;1* and several AMT genes (*OsAMT1;1,OsAMT2;2,OsAMT3;2,OsAMT3;3*) in roots and *OsAMT1;3* and *OsAMT3;3* in shoots exposed to LN (Table 3). Under MN, compared with uninfected tissues, the transcript levels of *OsNRT2;1* and *OsAMT3;3* in roots and *OsAMT3;3* in shoots were significantly elevated in infected rice (Table 3). At the S2 stage, the transcript levels of *OsAMT1;1, OsAMT2;2,* and *OsAMT3;3* in roots and *OsNRT2;1* and *OsAMT3;3* in shoots were elevated in infected compared with uninfected rice under LN and MN. At the S3 stage, transcription of *OsAMT2* and *OsAMT3;3* was induced by fungal infection in roots under LN (Table 3). However, there were not marked impacts caused by *P. liquidambari* on the expression levels of some genes (Table 3).

### Expression Levels of Genes Related in N Assimilation

The expression levels of genes in the *OsGOGAT, OsNR,* and *OsGS* gene families in rice were examined. As shown in Table 4, endophyte infection induced markedly higher expression levels of *OsNR1* in shoots under low nitrogen level, but they were not significantly increased in roots (Table 4). The effects of the endophyte on *OsNR1* expression levels were not markedly changed during exposure to middle and high nitrogen levels. Additionally, endophyte infection had no significant effects on the transcript levels of *OsNiR* (Table 4).

For the *OsGS* family, *P. liquidambari* infection largely upregulated the expression of *OsGS1;1* in roots and shoots at both the S1 and S2 stages (Table 4). For the *OsGOGAT* gene family, only the expression levels of *OsNADH-GOGAT* in rice roots were increased by endophyte treatment under LN at the S1 stage (Table 4). However, the endophyte did not significantly affect *OsNADH-GOGAT* transcription under MN or HN during other growing stages.

### Effects of *P. liquidambari* Infection on Phytohormone Levels in Rice

We examined the hormone content in infected and uninfected rice plants during exposure to three N levels to further investigate whether the effect caused by *P. liquidambari* infection involved the regulation of hormone levels in rice. We found that both shoots and roots of infected rice had higher levels of IAA and CTK at the S1 and S2 stages under LN and MN compared with uninfected plants (Fig. 3a, c); however, this effect was largely limited by the N fertilizer level and plant growth stage because there was no apparent difference between infected and uninfected plants under HN or at the S4 stage. In addition, the content of ETH in infected plants was higher than that in uninfected plants at the S3 stage (Fig. 3d). In contrast, there were no significant differences between

**Table 3** Expression levels of *OsNRT* and *OsAMT* in E+ and E− rice


| Gene | S1 | | | | | | S2 | | | | | | S3 | | | | | | S4 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LN/E+ | | MN/E+ | | HN/E+ | | LN/E+ | | MN/E+ | | HN/E+ | | LN/E+ | | MN/E+ | | HN/E+ | | LN/E+ | | MN/E+ | | HN/E+ | |
| name | Root | Shoot | Root | Shoot | Root | Shoot | Root | Shoot | Root | Shoot | Root | Shoot | Root | Shoot | Root | Shoot | Root | Shoot | Root | Shoot | Root | Shoot | Root | Shoot |
| *OsNRT1;1* | 0.322 | 0.224 | 0.275 | 0.163 | 0.437 | 0.133 | -0.246 | -0.337 | 0.108 | 0.115 | -0.025 | 0.137 | -0.322 | -0.156 | 0.145 | 0.107 | 0.120 | -0.036 | 0.152 | 0.056 | 0.148 | -0.075 | 0.133 | 0.136 |
| *OsNRT1;2* | 0.203 | 0.114 | 0.152 | 0.16 | 0.147 | 0.156 | 0.305 | 0.218 | 0.143 | 0.211 | 0.136 | 0.054 | 0.215 | 0.137 | 0.255 | 0.196 | -0.068 | 0.122 | 0.205 | 0.116 | 0.124 | -0.021 | 0.033 | 0.106 |
| *OsNRT2;1* | 0.644 | 0.192 | 0.572 | 0.167 | 0.144 | 0.096 | 0.369 | 0.254 | 0.533 | 0.109 | -0.122 | 0.037 | 0.26 | -0.112 | 0.163 | 0.208 | 0.133 | 0.131 | 0.132 | 0.058 | -0.028 | -0.103 | -0.097 | 0.155 |
| *OsAMT1;1* | 0.426 | 0.371 | 0.326 | 0.259 | 0.146 | 0.169 | 0.426 | 0.155 | 0.306 | 0.156 | 0.183 | 0.144 | 0.177 | -0.256 | 0.275 | -0.072 | 0.116 | 0.07 | 0.133 | 0.125 | 0.136 | 0.11 | 0.108 | 0.112 |
| *OsAMT1;2* | 0.141 | -0.094 | 0.101 | -0.083 | 0.096 | 0.113 | -0.155 | -0.205 | 0.107 | 0.151 | 0.072 | -0.057 | 0.104 | -0.11 | 0.065 | 0.103 | -0.315 | -0.143 | 0.031 | 0.093 | 0.146 | 0.106 | 0.122 | 0.154 |
| *OsAMT1;3* | 0.383 | 0.408 | 0.205 | 0.182 | 0.124 | 0.133 | 0.208 | 0.177 | -0.032 | -0.103 | 0.114 | 0.126 | 0.215 | 0.025 | 0.146 | 0.103 | 0.115 | 0.154 | 0.204 | 0.066 | 0.213 | 0.169 | 0.117 | 0.054 |
| *OsAMT2;1* | ND | 0.125 | ND | 0.107 | ND | 0.097 | 0.114 | 0.065 | 0.211 | 0.166 | 0.123 | 0.105 | ND | ND | ND | 0.169 | 0.158 | 0.127 | 0.045 | -0.129 | -0.083 | -0.072 | 0.029 | 0.104 |
| *OsAMT2;2* | 0.855 | 0.167 | 0.351 | 0.112 | 0.118 | 0.087 | 0.761 | 0.201 | 0.327 | -0.044 | 0.051 | 0.122 | 0.436 | 0.104 | 0.225 | ND | 0.117 | 0.146 | 0.136 | 0.147 | 0.155 | 0.125 | 0.164 | 0.121 |
| *OsAMT2;3* | -0.075 | ND | -0.092 | ND | 0.114 | ND | ND | ND | ND | ND | ND | ND | 0.145 | 0.071 | -0.091 | 0.038 | 0.106 | 0.133 | ND | ND | -0.223 | -0.146 | -0.032 | -0.255 |
| *OsAMT3;1* | -0.129 | -0.103 | 0.108 | 0.122 | -0.107 | -0.071 | 0.048 | -0.132 | 0.142 | 0.16 | -0.116 | 0.107 | 0.126 | 0.014 | 0.152 | 0.112 | -0.047 | 0.025 | ND | ND | ND | ND | ND | ND |
| *OsAMT3;2* | 0.512 | 0.186 | 0.327 | 0.153 | 0.105 | 0.114 | 0.329 | 0.211 | 0.244 | 0.102 | 0.135 | 0.115 | 0.241 | 0.115 | 0.148 | 0.036 | 0.104 | 0.105 | 0.168 | 0.122 | 0.119 | 0.147 | 0.089 | 0.106 |
| *OsAMT3;3* | 0.989 | 0.942 | 0.805 | 0.5 | -0.322 | 0.128 | 0.768 | 0.89 | 0.822 | 0.367 | 0.157 | 0.117 | 0.455 | 0.308 | -0.122 | 0.053 | 0.144 | 0.126 | 0.317 | 0.109 | 0.076 | 0.038 | 0.173 | 0.125 |

Mean values are shown on a log2 scale. The values of E- is zero. Values are means for three biological replicates. Color is used to visualize the data; pink indicates a higher expression level compared with E-, blue indicates a lower expression level compared with E-, gray indicates that the sample expression levels are too low to be detected (ND). The deeper color indicates the more significant differences between E + and E-. *LN* low nitrogen, *MN* middle nitrogen, *HN* high nitrogen, *E+* endophyte-infected, *E-*, endophyte-uninfected

**Table 4** Expression levels of genes in *OsGOGAT, OsNR,* and *OsGS* gene family


| Gene | S1 | | | | | | S2 | | | | | | S3 | | | | | | S4 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LN/E+ | | MN/E+ | | HN/E+ | | LN/E+ | | MN/E+ | | HN/E+ | | LN/E+ | | MN/E+ | | HN/E+ | | LN/E+ | | MN/E+ | | HN/E+ | |
| name | Root | Shoot | Root | Shoot | Root | Shoot | Root | Shoot | Root | Shoot | Root | Shoot | Root | Shoot | Root | Shoot | Root | Shoot | Root | Shoot | Root | Shoot | Root | Shoot |
| *OsNR1* | 0.216 | 0.422 | 0.172 | 0.182 | 0.096 | 0.107 | 0.346 | 0.225 | 0.144 | 0.156 | -0.046 | 0.122 | 0.233 | 0.281 | -0.133 | -0.098 | 0.115 | 0.127 | 0.255 | 0.192 | 0.270 | 0.043 | 0.125 | 0.182 |
| *OsNiR* | 0.106 | ND | -0.102 | ND | ND | ND | 0.209 | 0.107 | 0.078 | -0.126 | 0.13 | 0.113 | 0.075 | 0.106 | 0.17 | -0.141 | 0.125 | 0.12 | 0.144 | -0.056 | 0.103 | 0.064 | -0.138 | -0.105 |
| *OsGS1;1* | 0.634 | 0.325 | 0.462 | 0.344 | 0.226 | 0.145 | 0.549 | 0.622 | 0.362 | 0.215 | 0.158 | 0.163 | 0.28 | 0.134 | 0.155 | 0.064 | 0.09 | 0.117 | 0.301 | 0.215 | 0.089 | 0.133 | 0.047 | -0.048 |
| *OsGS1;2* | 0.125 | 0.106 | 0.115 | 0.127 | 0.102 | 0.133 | ND | ND | ND | ND | ND | ND | -0.156 | -0.098 | 0.133 | 0.126 | 0.034 | 0.082 | 0.145 | 0.237 | 0.116 | -0.116 | 0.126 | 0.18 |
| *OsGS2* | 0.141 | 0.306 | 0.128 | 0.124 | 0.118 | 0.115 | 0.244 | 0.116 | 0.187 | 0.084 | 0.103 | 0.107 | 0.147 | 0.12 | 0.117 | 0.106 | 0.154 | 0.178 | 0.131 | 0.134 | -0.061 | 0.149 | 0.145 | 0.107 |
| *OsNADH-GOGT* | 0.416 | 0.126 | 0.131 | 0.116 | 0.102 | 0.123 | 0.231 | 0.267 | 0.155 | 0.197 | 0.065 | 0.105 | 0.056 | 0.322 | 0.167 | 0.278 | -0.126 | -0.103 | 0.208 | 0.102 | 0.035 | 0.062 | 0.13 | 0.044 |
| *OsFd-GOGAT* | -0.092 | -0.103 | 0.122 | 0.144 | 0.106 | 0.112 | 0.122 | 0.098 | 0.142 | -0.13 | 0.046 | 0.117 | ND | ND | ND | ND | 0.177 | 0.132 | ND | ND | ND | ND | ND | ND |

Mean values are shown on a log2 scale. The values of E− is zero. Values are means for three biological replicates. Color is used to visualize the data; pink indicates a higher expression level compared with E−, blue indicates a lower expression level compared with E−, and gray indicates that the sample expression levels are too low to be detected (ND). The deeper color indicates the more significant differences between E+ and E−. *LN* low nitrogen, *MN* middle nitrogen, *HN* high nitrogen, *E+* endophyte-infected, *E−* endophyte-uninfected

infected and uninfected plants at any stage of growth. At the S1 stage, compared with uninfected plants, the content of IAA in rice shoots and roots treated with *P. liquidambari* was enhanced by 37.06 and 23.38% under LN and by 9.56 and 9.54% under MN, respectively; the content of CTK in *P. liquidambari*-treated rice shoots and roots was enhanced by 22.73 and 21.72% under LN and MN by 59.17 and 12.86%, respectively. At the S2 stage, there was a mean increase in IAA of 30.11 and 24.66% in rice shoots and roots under LN, respectively, compared with

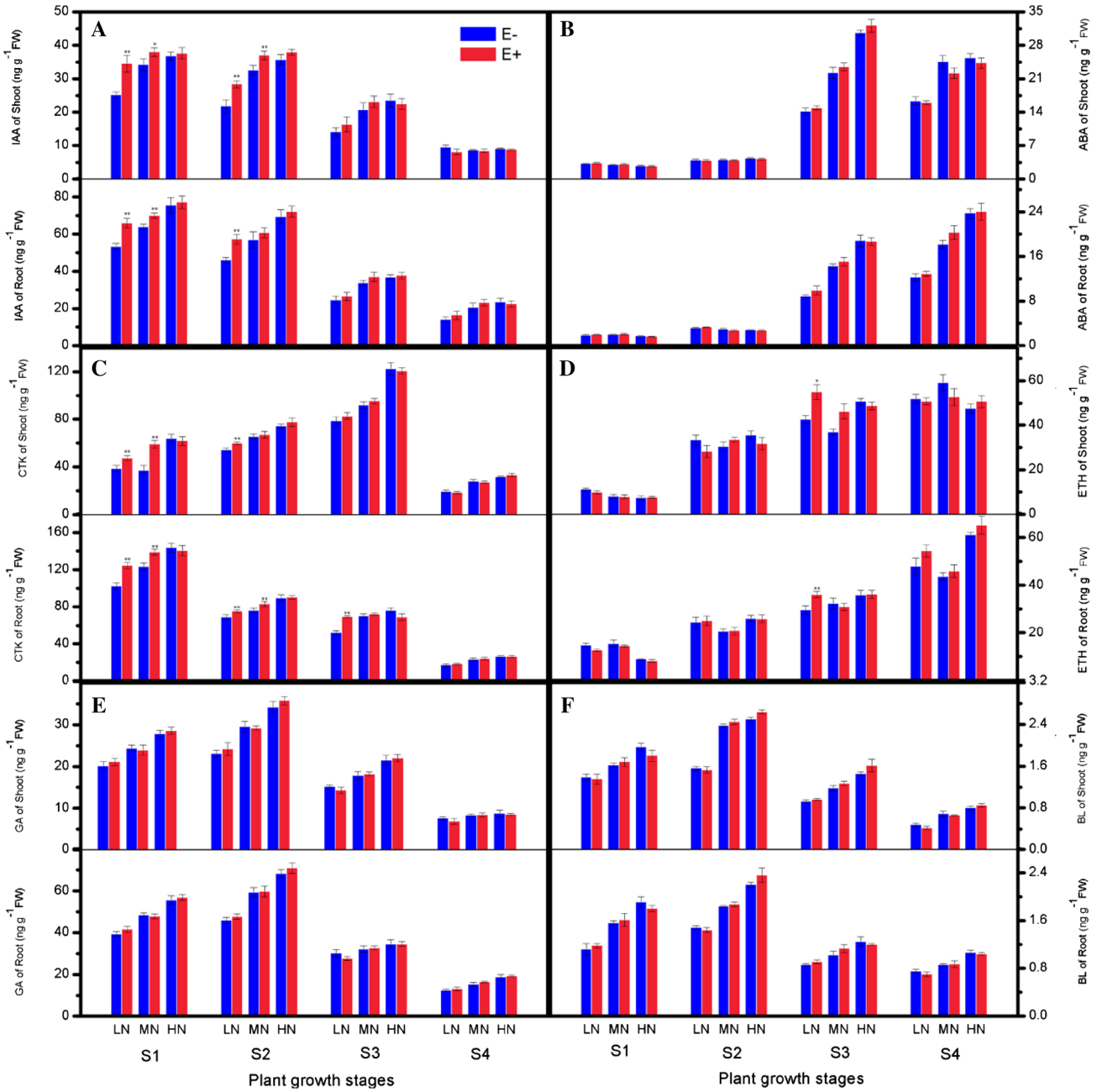


**Fig. 3** Content of six phytohormones, including **a** auxin (IAA), **b** abscisic acid (ABA), **c** cytokinin (CTK), **d** ethylene (ETH), **e** gibberellin (GA), and **f** brassinolide (BL) in *P. liquidambari*-infected (E+) and uninfected rice (E−). Values are means for three biological replicates. *Bars* denote the standard error of the mean. *Asterisks* indicate significant differences (*$P<0.05$; **$P<0.01$). *LN* low nitrogen, *MN* middle nitrogen, *HN*, high nitrogen, *E+* endophyte-infected, *E−* endophyte-uninfected. (Color figure online)

uninfected plants, and the content of IAA in *P. liquidambari*-treated rice shoots and roots was increased by 37.06 and 23.38% under LN and by 13.67% in roots under MN. The content of CTK in *P. liquidambari*-treated rice shoots and roots increased by 2 9.94 and 9.18% under LN, and by 59.17% in roots under MN. At the S3 stage, *P. liquidambari* infection led to a significant increase in ethylene by 23.71 and 29.21% in shoots and roots under LN, respectively, and by 9.75% in roots under MN. There were no apparent endophyte effects on the contents of the other hormones (ABA, BL, and GA) during the four growth stages (Fig. 3b, e, f).

### Effects of Exogenous Phytohormones and their Specific Inhibitors on Nitrogen Contents in Rice

To verify whether the regulation of endophyte infection on the level of phytohormones in rice was involved in the beneficial effects of endophyte infection on N metabolism and N uptake in rice, exogenous hormones (IAA, zeatin, and ACC) and their specific inhibitors (PCIB, lovastatin, and AOA) were applied. As shown in Fig. 4, the application of IAA (10 μM) significantly increased the concentration of $NH_4^+$-N and total N in shoots by 89.68 and 55.05%, respectively, and the concentration of $NO_3^-$-N, $NH_4^+$-N, and total N increased by 24.76, 22.8, and 63.58%, respectively. Similarly, the contents of $NO_3^-$-N, $NH_4^+$-N, and total N in rice shoots and roots were enhanced substantially by the application of exogenous zeatin (1 μM) (Fig. 5). The $NH_4^+$-N and total N concentrations were increased in the shoots and roots of rice treated with ACC (100 μM), but the $NO_3^-$-N concentration was slightly decreased (Fig. 6). Consistent with the previous results, the application of inhibitors (PCIB, 50 μM; AOA, 50 μM) resulted in a mean decrease in $NH_4^+$-N and total N content of 43.52, 54.85, 59.62, and 46.32% in rice shoots and roots, respectively. Additionally, application of lovastatin (40 μM) led to a significant decrease in N content in rice shoots and roots. Pre-experimental results revealed that a high concentration of inhibitor (PCIB, 100 μM; AOA, 100 μM; lovastatin, 100 μM) had negative effects on rice, in which the content of N deceased and growth and development were repressed. A low inhibitor concentration (PCIB, 10 μM; AOA, 10 μM; lovastatin, 10 μM) only slightly suppressed the accumulation of N, and no significant differences were observed. Moreover,

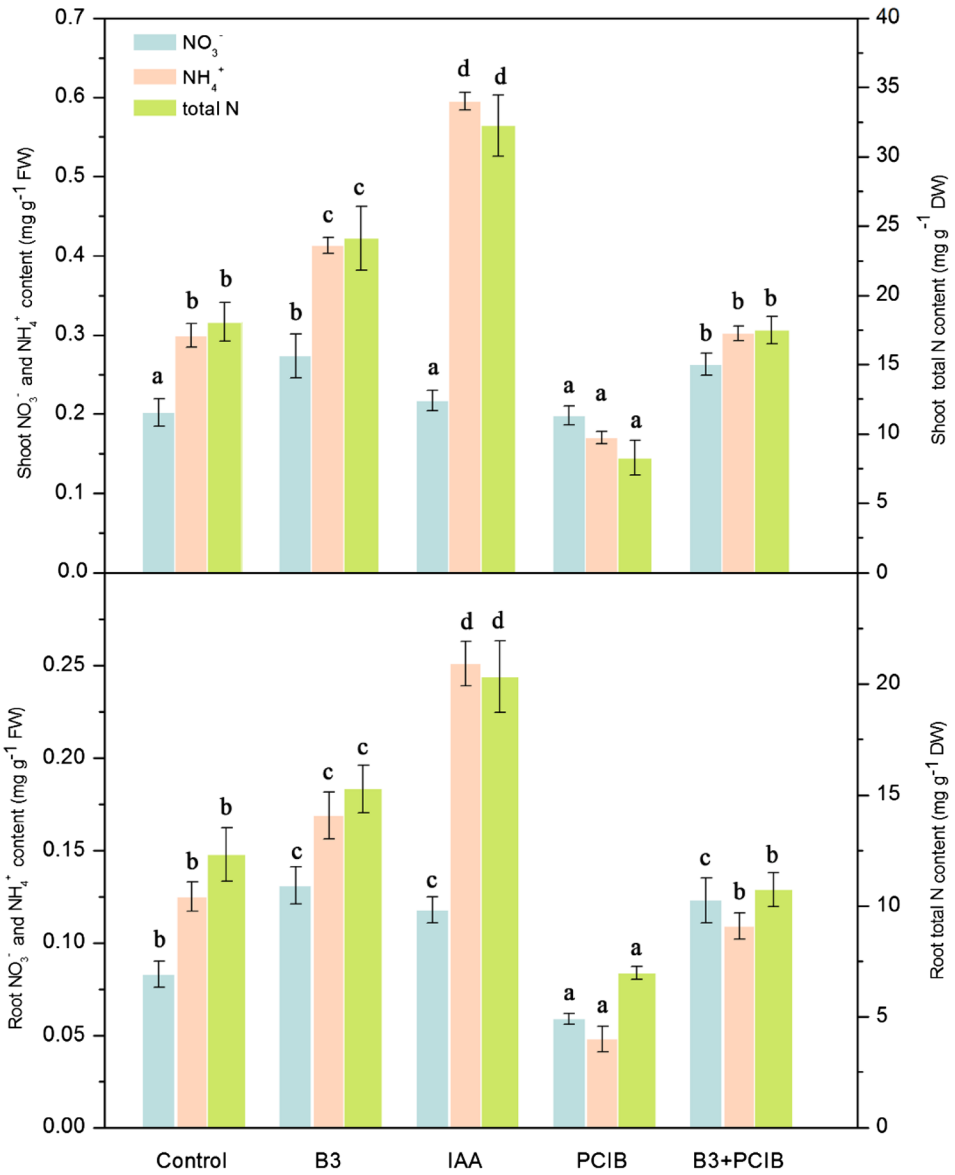


**Fig. 4** Effect of *P. liquidambari* infection and the application of IAA and inhibitor (50 μM PCIB) on the contents of $NO_3^-$-N, $NH_4^+$-N, and total N. Inhibitor was added 1 day prior to *P. liquidambari* inoculation. *Bars* denote the standard error of the mean. Values are means for three biological replicates. *Bars* with *different lower case letters* indicate significant differences between different treatments ($P < 0.01$). (Color figure online)

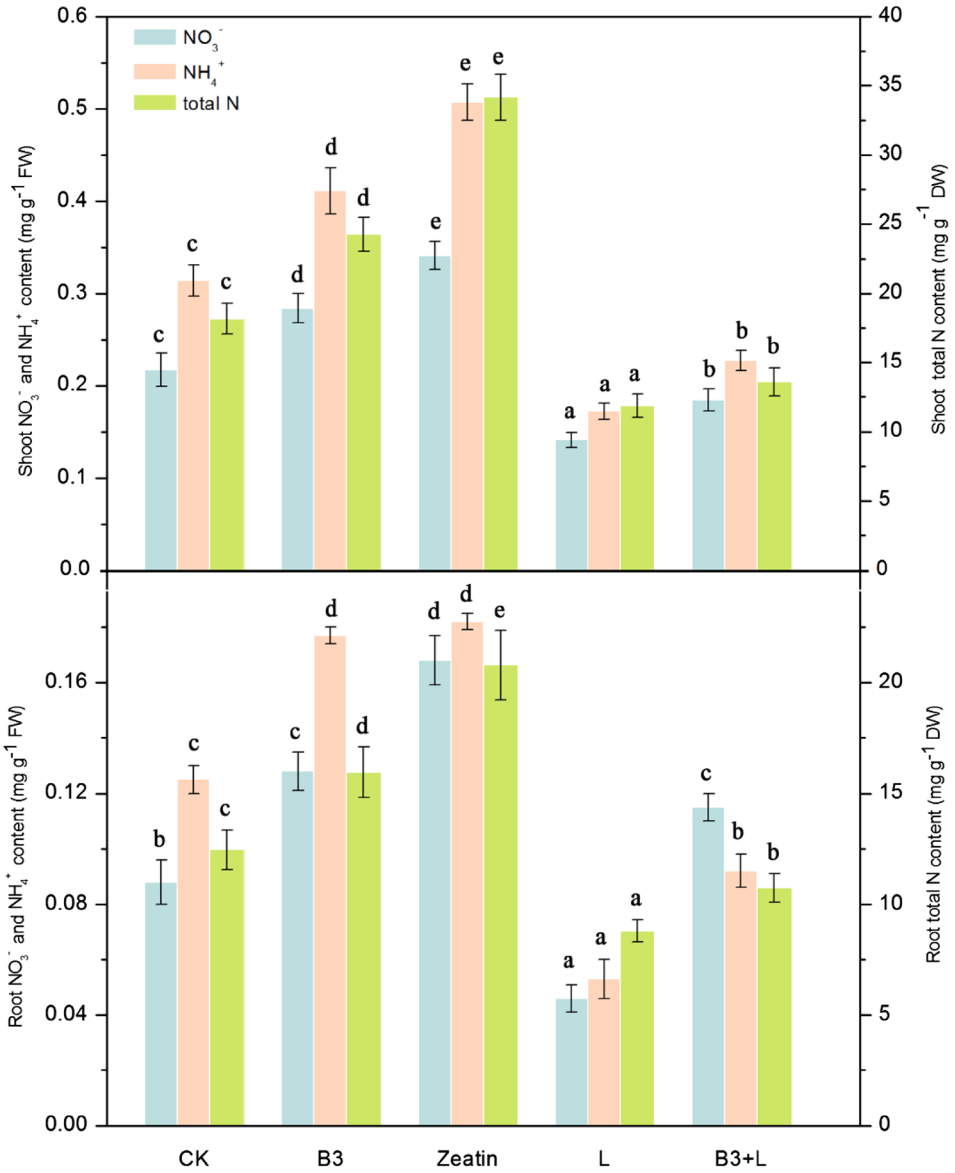


**Fig. 5** Effect of *P. liquidambari* infection and application of zeatin and inhibitor (50 μM Lovastatin) on the contents of $NO_3^-$-N, $NH_4^+$-N, and total N. Inhibitor was added 1 day prior to *P. liquidambari* inoculation. *Bars* denote the standard error of the mean. Values are means for three biological replicates. *Bars* with *different lower case letters* indicate significant differences between different treatments ($P < 0.01$). (Color figure online)

*P. liquidambari* infection could, to some extent, alleviate the negative effect of inhibitor application (PCIB, 100 μM; AOA, 100 μM; lovastatin, 100 μM) on N content in rice. These results indicated that *P. liquidambari* symbiosis may modulate the content of IAA, ETH, and CTK to improve the level of N metabolism and N uptake in host rice.

## Discussion

### N Accumulation and Metabolic Level in Response to *P. liquidambari* Symbiosis

It is well-known that endophyte fungi can establish symbioses with many plants, and their capacity to influence some important ecosystem processes, including plant diversity, plant-herbivore interactions, and plant productivity make them the focus of research (van der Heijden and others 2006). Fungal endophytes have been frequently reported to have beneficial effects on their host plants, such as they supply nutrients to their host plants (Rodriguez and others 2008). In this study, we used the rice cultivar "Wuyunjing 23" in 2015, and our results showed *P. liquidambari* in rice substantially altered N uptake and N metabolism, promoted rice growth, and increased NUE and the productivity of rice (Table 2). Similarly, the results of Yang and others (2015) and Siddikee and others (2016), who used "Wuyunjing 7" in 2013 and 2015, respectively, also showed that *P. liquidambari* had similar useful impacts on rice nitrogen uptake and metabolism. These findings indicated that the improvements provided by *P. liquidambari* were stable among different rice cultivars and growing years. Remarkably, these

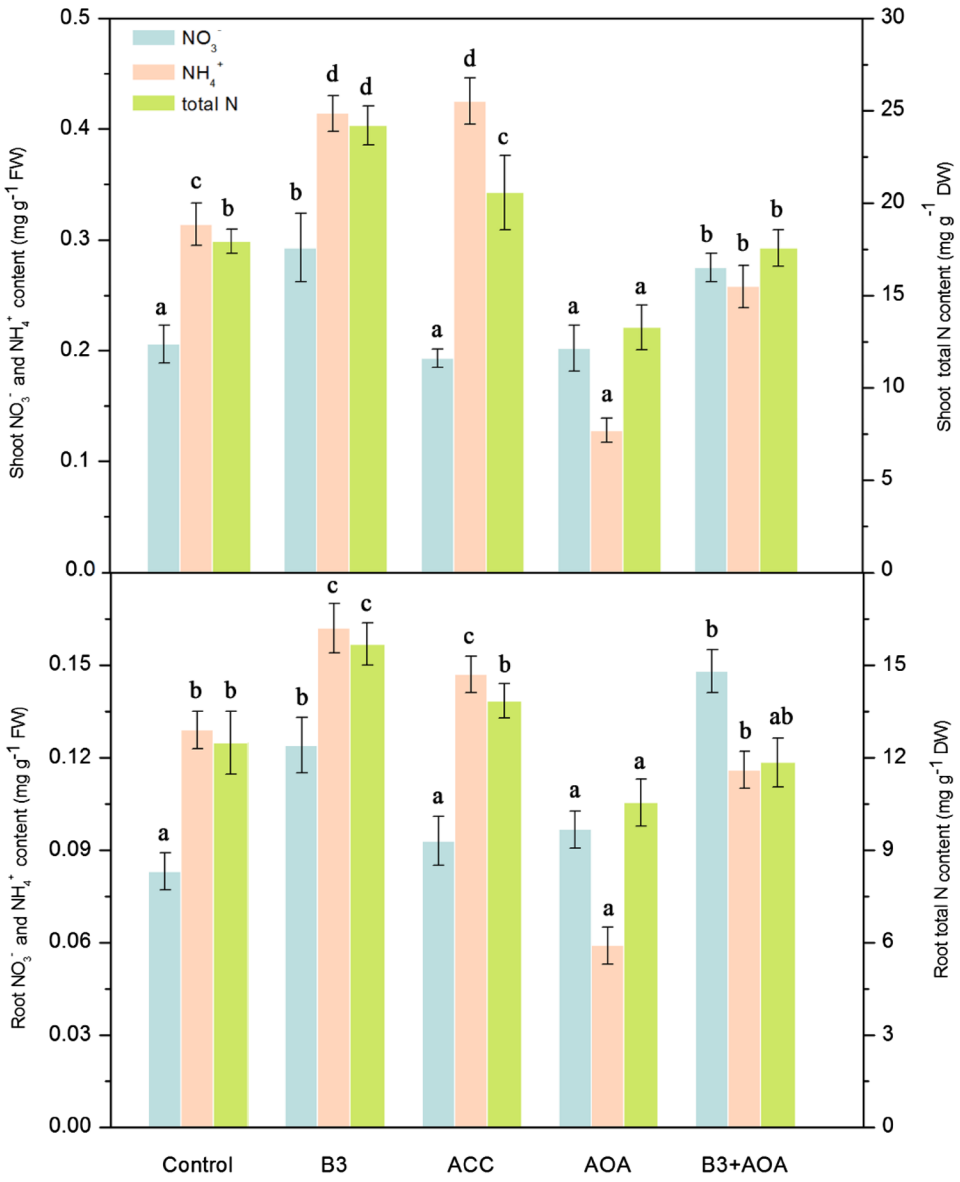


**Fig. 6** Effect of *P. liquidambari* infection and application of ACC and inhibitor (50 μM AOA) on the contents of $NO_3^-$-N, $NH_4^+$-N, and total N. Inhibitor was added 1 day prior to *P. liquidambari* inoculation. *Bars* denote the standard error of the mean. Values are means for three biological replicates. *Bars* with *different lower case letters* indicate significant differences between different treatments ($P < 0.01$). (Color figure online)

effects normally occurred when supplied with low nitrogen levels. Yong Li and others (2011) found that the nitrogen use efficiency of rice seedlings decreased under high nitrogen supply. The research of Azcón and others (2008) showed that N fertilization level can influence the nitrogen absorption of plants by the fungal symbiont, for instance, mycorrhizal plants can modulate plant nitrogen acquisition during exposure to different nitrogen levels in the soil. Similarly, Upson and others (2009) reported that endophytes can increase the NUE of young plants in N-depleted soils.

The availability of N often limits plant growth (Yoneyama and others 2007). There is increasing evidence that fungal endophytes are involved in N metabolism. Our results showed that the mean activities of NR and GS in rice shoots and roots are increased by the *P. liquidambari* symbiont under the low nitrogen level throughout the various rice growth stages, excluding the ripening stage (Figs. 1, 2). Similar results were reported by Sherameti and others (2005), who showed that the endophytic fungus *P. indica* stimulated N accumulation by enhancing the expression of NR in plant roots. We used pot experiments to quantify the transcript levels of N metabolism-relevant genes, such as *OsNR, OsGS,* and *OsGOGAT*GS, to identify whether the changes caused by *P. liquidambari* were related to NR or GS transcription in the four growth stages of rice under field conditions. The qRT-PCR results showed that N metabolism-relevant genes (*OsNR1, OsGS1, OsGS2*, and *OsNADH-GOGAT*) were up-regulated in *P. liquidambari*-infected plants under low N concentrations in stages S1 and S2 (Table 4). Our results are consistent with those of Yang and others (2013), who used hydroponic experiments to assay the transcript levels of the above genes at

the seedling stage. In addition, a previous study by Yang and others (2013, 2014) showed that the contents of total N, $NH_4^+$, and $NO_3^-$ in endophyte-infected plants were significantly higher than those of uninfected plants, indicating that the metabolic level of N in rice plants was substantially altered by *P. liquidambari*.

Plants take up ammonium and nitrate and transport them to plant tissues using AMT protein family members and NRT protein family members, respectively. Endophytic fungi have frequently been considered to be involved in N transfer. In the present study, endophyte-infected plants displayed a higher total N concentration than uninfected plants (Table 1), suggesting that endophyte infection promotes the uptake and assimilation of more N in infected compared with uninfected rice. Analyses of gene transcript levels in rice using pot experiments revealed that *P. liquidambari* infection lead to a substantial increase of expression levels of some *OsNRT* and *OsAMT* gene family members in shoots or roots under field conditions of the four growth stages of rice. Compared with uninfected rice tissues, endophyte-infected rice had higher transcript levels of the genes, including, *OsAMT3;3, OsAMT1;1, OsAMT1;3, OsAMT2;2, OsAMT3;2,* and *OsNRT2;1*. The most apparent improvement in their expression levels caused by *P. liquidambari* was mainly observed under low nitrogen conditions in the S1 and S2 stages (Table 3). The above results are similar to those of Yang and others (2013), who used hydroponic experiments to assay the transcript levels of the above genes at the seedling stage. Likewise, Koegel and others (2013) found that the expression of plant ammonium transporters, *SbAMT3;1* and *SbAMT4*, was induced only in cells of the arbuscule in sorghum (*Sorghum bicolor*).

### *P. liquidambari* Regulates Phytohormones to Promote N Uptake and Metabolism in Rice

Some endophytic fungi can increase the growth and fitness of host plants by increasing phytohormones, such as cytokinin, ethylene indole-3-acetic acid, and indole-3-acetonitrile (Waqas and others 2012; Barnawal and others 2015). In the present study, we determined the content of six phytohormones in endophyte-infected rice and endophyte-uninfected rice under our experimental conditions, and the results revealed that endophyte *P. liquidambari* infection can increase the content of IAA and CTK in the S1 stage under low N levels (Fig. 3a, c), and enhance the concentration of ethylene in the S3 and S4 stages (Fig. 3d). In the present study, the content of phytohormone we detected was the total content of phytohormone in endophyte fungi-rice symbiosis; however, the source of the enhanced IAA and CTK level is not known. Increasing evidence shows that endophytes can produce different plant hormones (Waqas and others 2012; Cassán and others 2013) or regulate the production of hormones (ethylene, auxin, and cytokinin) in host plants (Singh and others 2013). Moreover, the previous study showed that *P. liquidambari* can produce 3-indole acetic acid in vitro, however CTK was not detected (Chen and others 2011). Thus, we speculate that the enhanced IAA level may be from the sum of the *P. liquidambari* source and the rice source, and the enhanced CTK may be from the rice source. We will further determine the source of hormone by molecular and physiological methods.

Auxin is considered to mediate N signals from shoots to roots (Fukaki and Tasaka 2009). NRT1.1 and NRT2.1, two of the main transporters of nitrate uptake, are hormone-responsive genes. The results of our pot experiments revealed that endophyte-infected rice had a significantly higher level of IAA in different tissues, and these findings were consistent with the results of the hydroponic analysis, which revealed that the contents of free ammonium and total nitrogen in rice were increased by exogenous IAA and decreased by PCIB and that the application of endophyte alleviated the suppression by PCIB (Fig. 4). It is well known that CTKs participate in some important processes of plant growth and development, including nitrogen signaling. Previous research indicated that the N level and CTK contents are closely linked in *Urtica dioica*, tobacco; in *Plantago major*, exogenous CTK application can relieve the negative impacts caused by a N- deficient condition on plant growth to some degree (Kiba and others 2011). Most *AtNRTs* expressed in shoots are up-regulated by CTK under both LN and HN conditions (Kiba and others 2011). The above results support an interaction of the CTK signaling pathways and $NO_3^-$ in the control of NR. In this study, similar to the results obtained for IAA, the application of exogenous zeatin increased the levels of $NO_3^-$, $NH_4^+$, and total N in rice, and the inhibitor lovastatin repressed the accumulation of N in rice. Similarly, endophyte infection weakened the repression caused by the inhibitor (Fig. 5). Ethylene plays crucial regulatory roles in plant responses to the availability of mineral nutrients such as nitrogen (Iqbal and others 2015; Khan and others 2015) and in control of plant responses under both optimal and stressful conditions (Iqbal and others 2013). It has also been shown that the application of exogenous ethylene increases N assimilation and photosynthesis in *Brassica juncea* plants under different levels of N (Khan and others 2008). Our results indicated that ethylene may be involved in the improvement of rice N uptake and metabolism caused by endophyte infection (Fig. 6).

Although this study did not demonstrate a significant effect on the levels of GA, ABA, and BL caused by endophyte infection, it does not mean that GA, ABA, and BL do not participate in the N use efficiency improvement in rice caused by the endophyte *P. liquidambari* because hormone

signaling systems build a network and mutually regulate signaling, transport, and metabolic systems. The cross-talk between auxin and ethylene has been well defined. It is reported that the interaction between auxin and ethylene participates in PGPR *Burkholderia phytofirmans* PsJN promoting the growth of *Arabidopsis thaliana* (Poupin and others 2016). So we propose that *P. liquidambari* and nitrogen are linked by a multilevel, complicated cycle that controls plant growth, development, and nitrogen metabolism by regulating phytohormonal level, and that there is interaction between plant hormones that encompasses synergism and antagonism to avoid an independent effect on N use.

### *P. liquidambari* Shortens the Growth Period of Rice

Plant phenology is a very important aspect of plant ecology including development, reproduction, flowering time of plant, and ripe of grain (Cleland and others 2007; Panke-Buisse and others 2015). Interestingly, compared with uninfected rice, the phenology of rice was changed by *P. liquidambari* infection in that the growth period of *P. liquidambari*-infected rice was shortened by approximately 10 days and the flowering time was 4 days earlier. It is well-known that plant phenology depends on many different environmental variables. There is increasing evidence that phytohormones participate in the regulation of reproduction, for example, IAA, CTK, GA, and ABA are likely to participate in the regulation of processes related to rice panicle initiation and grain filling. Tadiello and others (2016) showed that ethylene-auxin cross-talk is involved in peach ripening. Our results showed that the contents of IAA and CTK in the S1 and S2 stages (Fig. 3a and 3c) and the content of ETH in the S3 stage (Fig. 3d) was regulated by *P. liquidambari* infection. Another factor is soil microbial communities which have rarely been acknowledged as probable drivers of flowering time. Flowering time is an important ecological trait for plants and contributes to rice yield. Previous studies investigating the relationship between the soil microbiome and flowering time, used domesticated plants, artificial microbial communities, and/or biota from heavily disturbed soils (Lau and Lennon 2011). These experiments provide evidence that soil microbes alter plant reproductive timing and selection pressures. Panke-Buisse and others (2015) reported a high level of soil microbiome reproducibility in altering plant flowering time and soil functions. A previous study indicated that *P. liquidambari* colonization affected nitrogen transformation processes and related microorganisms in the rice rhizosphere (Yang and others 2015). So the alteration of microbial communities in the rice rhizosphere caused by *P. liquidambari* may be one of the potential mechanisms of the changes of plant phenology induced by *P. liquidambari*.

In addition, the availability of limited nutrients also affects the reproduction of plants. Plants exposed to nitrogen deficient conditions may display an earlier reproductive stage, leading to earlier senescence (Ren and others 2013). In contrast, the reports of Panke-Buisse and others (2015) showed that low N or P levels may lead to a reproductive delay and a mean increase of biomass in *A. thaliana*.

However, the metabolic effect of *P. liquidambari* infection during the rice growth period is largely unknown. Based on the evidence obtained, we conclude that *P. liquidambari* can affect the phenology of rice by regulating hormone levels or altering the quality and quantity of root exudates released into the soil to influence the structure and activity of the soil microbial community under LN.

## Conclusions

Our results showed that successful colonization of *P. liquidambari* in rice substantially altered N use, induced the expression of some genes related to N uptake and metabolism, promoted rice growth, and increased NUE and the productivity of rice. Moreover, the contents of auxin (IAA), cytokinin (CTK), and ethylene (ETH) in the host rice were remarkably changed by *P. liquidambari* infection under low nitrogen levels indicating that *P. liquidambari* symbiosis may regulate the contents of IAA, ETH, and CTK to improve N metabolism and uptake in host rice, especially in N-limited soils. Our work emphasizes the important role of endophyte fungi in plant nutrient absorption under N-deficient conditions. The endophyte fungi, *P. liquidambari*, may be a good candidate for decreasing nitrogen fertilizer use and improving plant yield in sustainable agriculture.

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**Compliance with Ethical Standards**

**Conflict of interest** The authors declare that they have no conflict of interest and the publication of the work has been approved by all co-authors.

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