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The effect of nitrogen level on rice growth, carbon-nitrogen metabolism and gene expression

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Abstract: As one of the essential macroelements, nitrogen (N) plays an important role in plant growth and development. In order to know the effect of different N levels on the rice plant growth and carbon-nitrogen metabolism, we analyzed the rice growth phenotype, leaf SPAD value, photosynthesis, carbon-nitrogen metabolic status and gene expression profile under four different N levels (0×N, 0.1×N, 1×N and 5×N). The plant height and dry weight increased with increasing N levels, whereas an opposite trend was observed for the root length, which decreased with increasing N levels. The leaf SPAD value, stem nitrate concentration, soluble proteins, photosynthetic rate, stomatal conductance and total nitrogen concentration increased with increasing N levels, whereas an opposite trend was observed for soluble carbohydrates and carbon/nitrogen ratio which decreased with increasing N levels. Metabolite profile analysis revealed that the low N treatment caused visible decreases in the concentrations of total sugars and organic acids in the leaves, while caused visible increases in the concentrations of total sugars, organic acids and free amino acids in the roots. Gene expression analysis showed that the transcriptional levels of 5 genes (*GS1;3*, *NADH-GOGAT1*, *NADH-GOGAT2*, *PEPC4* and *PEPC7*) altered significantly under four different N levels.

Key words: rice; carbon; gene expression; metabolite profile; nitrogen.

Introduction

Nitrogen (N) is one of the essential macroelements required for plant growth and development. It is the constituent of key cell molecules such as amino acids, nucleic acids, chlorophyll, ATP and several plant hormones, which are involved in many biological processes, including carbon metabolism, amino acid metabolism, photosynthesis and protein production (Frink et al. 1999; Crawford & Forde 2002). N deficiency may lead to dramatic changes in plant growth and development, such as root branching, increased root to shoot ratio, old leaf chlorosis, reduced growth and photosynthesis, fewer seed production (Stitt & Krapp 1999; Good et al. 2004; Ding et al. 2005; Diaz et al. 2006). In addition, carbon (C) is also crucial for the routine and fundamental cellular activities in plants. Carbon compounds, in particular sucrose, glucose and organic acids, provide both the energy and the C skeletons for ammonium (NH_4^+) assimilation during amino acid biosynthesis. Amino acids and the resulting proteins, in particular enzymes, are essential for almost all cellular activities, including various steps of the carbon and nitrogen metabolic reactions (Zheng 2009). Recently, it has been recognized that cellular C and N metabolism must be tightly coordinated. In addition to their independent utilization, the coordination and optimal functioning of the metabolic pathways for N and C assimilation in plants are critical for determining plant growth and, ultimately, biomass accumulation (Krapp et al. 2002; Krapp & Truong 2005). Therefore, maintaining an appropriate balance or ratio of carbohydrates to nitrogen metabolites in the cell, referred to as the "C/N balance", is also important for the regulation of plant growth, development and yield (Coruzzi & Zhou 2001; Martin et al. 2002; Zheng 2009; Nunes-Nesi et al. 2010).

In agriculture, crop production requires abundant N and which is generally the most common limiting factor for crop growth and yield. In order to meet the high production of crops, large amounts of synthetic N fertilizers are applied on arable land by farmers in China. In the last 50 years, there has been a dramatic increase in fertilizer applications in China. In 2002, China used 30% of N fertilizers produced worldwide, although its arable land accounts only for 10% of the world total (http://faostat. fao.org/). However, applications of large quantities of synthetic N fertilizers to increase crop yield are not economically sustainable and placed a heavy economic burden on the farmers, and also result to environmental pollutions. Crop plants use only less than half of the applied N fertilizers (Socolow 1999). Zhang et al. (2011) reported that the N use ef-

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ficiency of midseason rice in China is less than 30%, which means that nearly 70% of the N input is lost into the ecosystem and causes many problems, such as eutrophication, air pollution, water pollution, soil acidification, soil degradation and greenhouse gas (nitrous oxide) emission (Tilman et al. 2001; Galloway et al. 2008; Vitousek et al. 2009; Liu et al. 2010; Godfray 2010; Guo et al. 2010; Zhu & Chen 2010; Chen et al. 2011). Moreover, because plants grown under excess nitrogen conditions are more susceptible to lodging and pest damage, over application of N fertilizer often reduces rice grain yield and also partly accounts for the poor eating and cooking quality of the rice grains produced (Zhang 2007).

In order to know how rice responded to the different levels of environmental nitrogen, to understand the details about rice growth, carbon-nitrogen metabolites and genes expression patterns under both low and high nitrogen levels, particularly the combining analysis of nitrogen and carbon metabolic status, we analyzed the rice growth, leaf SPAD value, photosynthesis, carbon-nitrogen metabolic status and gene expression profile under four different nitrogen levels (no nitrogen, low nitrogen, normal nitrogen and high nitrogen).

Material and methods

Plant growth conditions

Seeds of Zhonghua 11 (*Oryza sativa* ssp. *japonica*) were germinated and sowed in sand. At the 3-leaf stage, the seedlings were transferred into the complete nutrient solution containing 1.44 mM NH_4NO_3, 0.3 mM NaH_2PO_4, 0.5 mM K_2SO_4, 1.0 mM $CaCl_2$, 1.6 mM $MgSO_4$, 0.17 mM Na_2SiO_3, 50 μM Fe-EDTA, 0.06 μM $(NH_4)_6Mo_7O_{24}$, 15 μM H_3BO_3, 8 μM $MnCl_2$, 0.12 μM $CuSO_4$, 0.12 μM $ZnSO_4$, 29 μM $FeCl_3$ and 40.5 μM citric acid, pH 5.5 (Yoshida et al. 1976). The culture solution was refreshed every 3 days. After a week, the plants were transferred into a culture solution without NH_4NO_3 as no nitrogen or 0×N treatment (little NH_4NO_3 in water), a culture solution with 1/10 NH_4NO_3 as low nitrogen or 0.1×N treatment (0.144 mM NH_4NO_3), a culture solution with 5 fold NH_4NO_3 as high nitrogen or 5×N treatment (7.2 mM NH_4NO_3), and culture solution with complete nutrients as normal nitrogen or 1×N treatment (1.44 mM NH_4NO_3). The plant materials were harvested at the tillering stage and the heading stage for analysis of the root length, plant height, dry weight, leaf SPAD (Soil and plant analyzer development) value, photosynthesis, stem nitrate concentration, carbon and nitrogen concentration, concentrations of soluble proteins and carbohydrates, metabolic profiling and gene expression patterns.

Determination of the physiological parameters

The flag leaf SPAD value and photosynthesis parameters were determined by a chlorophyll meter (SPAD-502) and a Li-6400XT portable photosynthesis system (USA, Li-COR), respectively. Ten plants were randomly selected, the average SPAD value and photosynthesis parameters of the upper, middle and bottom portion of each flag leaf was analyzed. The carbon and nitrogen concentration was determined by a C/N analyzer (Elementar, Vario MAX CN, German) according to the manufacturer's instructions, with L-glutamic acid as a standard. For the soluble protein and carbohydrate analysis, three samples of randomly mixed plant root, stem and leaf materials from three biological replicates were harvested. The plant materials were homogenized by grinding the freshly harvested materials on ice with extraction buffer [10 mM Trizma (pH 7.5), 5 mM sodium glutamate, 10 mM $MgSO_4$, 1 mM dithiothreitol, 10% (v/v) glycerol, and 0.05% (v/v) Triton X-100]. The homogenates were then centrifuged at 12,000 *g* for 20 min at 4℃ (Melo et al. 2003). The soluble protein concentration of the extract was measured by the Bradford (1976) protein assay using Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL, USA); bovine serum albumin was used as the standard protein. The soluble carbohydrates were extracted from pre-dried plant materials with boiling water and colorimetrically measured according to the anthrone procedure (Morris 1948; Maness 2010). For the metabolite profiling analysis, samples of six randomly mixed plant root and leaf materials were harvested and analyzed using the GC-TOF-MS method. Extracts from 200 mg fresh weight samples were used. The data pre-treatment and normalization, the alignments and the metabolite identification were performed as described by Kusano et al. (2007a; 2007b) and Redestig et al. (2009).

Gene expression analysis

For gene expression analysis, both root and leaf materials were harvested from three biological replicates under four different nitrogen levels at the tillering stage. Total RNA was extracted with TriZol reagent (Invitrogen, Germany) according to the manufacturer's instructions. For q-RT PCR analysis, first-strand cDNAs were synthesized from DNaseI-treated total RNA using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Q-RT PCR was performed in an optical 96-well plate with an ABI PRISM 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Each reaction contained 12.5 μL of 2×SYBR Green Master Mix reagent (Applied Biosystems), 3.0 μL of cDNA, and 200 μM each of the gene-specific primers in a final volume of 25 μL. The thermal cycle used was as follows: 95℃ for 3 min followed by 45 cycles of 95℃ for 30 s, 60℃ for 30 s, and 72℃ for 40 s. All gene-specific primers for q-RT PCR were designed on the basis of the cDNA sequences and listed in Table 1. The specific primer for the rice *actin* gene (AK070531) was used as an internal control. The primers were designed using Primer Express Software (Foster City, CA, USA) and checked using the BLAST program with the rice genomic sequence available in the database of the Institute for Genomic Research (TIGR, http://rice.plantbiology.msu.edu/) to ensure that the primers would amplify a unique and desired cDNA segment. The specificity of the reactions was checked by melting curve analysis, and three replicates of each cDNA sample were used for q-RT PCR analysis.

Results

Plant growth phenotypes under four different nitrogen levels

In order to test the effect of different nitrogen levels on the rice plant growth, we analyzed the root length, plant height, dry weight of the root, shoot and plant at both tillering and heading stages grown hydroponically under four different nitrogen levels (0×N, 0.1×N, 1×N and 5×N). The plant height and dry weight increased with increasing nitrogen levels, whereas an opposite trend was observed for the root length, which decreased with increasing nitrogen levels, particularly at

Table 1. Primer sequences of the key genes involved in the carbon and nitrogen metabolism used in qRT-PCR analysis.

Gene name	cDNA accession NO.	Primer sequence
NRT1;1	AK066920	F: CCTCGCAAGTGACCCTTGAAT R: CGATGGCTAATGAGGAACCCTT
NRT1;2	AK101480	F: GAACATGCGGATCATGTCGTT R: CGATCACGGAGCTGTACATGAG
NRT2	AK109733	F: TTCGCGAACCCGCATATGA R: GTTGAGGTTGTCGCGGATGAT
NR1	AK102178	F: ACTACCATTACCGCGACAACC R: CTCGTTTATCATGTACTCCGGC
NR2	AK121810	F: AGCTGAACGTGAACTCGGTGA R: AGGCGTATCCCTTCATGGTGT
GS1;1	AK109397	F: GAGTCGTCGTCTCATTTGACCC R: GTAGCCACCATCGTTCCTCATC
GS1;2	AK243037	F: TTTTCAAGGACCCGTTCAGGA R: CGGCACTGTGCCTCTTGTTAGT
GS1;3	AK099290	F: TCAAGCCATCTTCAGAGACCCA R: TACCGGTTGTTCGTCGGAATC
GS2	AK063706	F: AGGATCGGACAAATCGTTTGG R: GCATGACCTCTCCATTTGTTCC
Fd-GOGAT1	AK102025	F: AAATGCCTCTTTGCAAGGCC R: GACTGTGAG CCCCATCCAAATA
Fd-GOGAT2	AK068130	F: CCGATGCGATTGAGAATGAGA R: CTTCTTGGCAATGACACCTGC
NADH-GOGAT1	AK105755	F: TGCTTGAGAGAATGGCGCA R: AACCCAGCATCCTTTGTCACC
NADH-GOGAT2	AK070485	F: GGTTGTCATTGGTGGTGGAGA R: TGGTGGCTCTGGCAAAAGTT
RUBISCO	AK243615	F: AGGCTTCAAATTGCCGTTGA R: TCTAGGCCATCCAGTTCCTCCT
PEPC1	AK100688	F: ACATTCCGTGTTGCTGCAGAG R: TGCAACAGTTCAACCGCTAGG
PEPC2	AK066635	F: CAGAAGCACGCAAGCATTAGG R: CGCGAGAATCTCTCTCTGAAGG
PEPC3	AK101274	F: ACCGGTCCATTGTCTTCCAAG R: CGTTTTGATGGCCTACTTCCAA
PEPC4	AK065425	F: TGGATGAGATGGCTGTTGTGG R: TTCTGTCTCAGGTGTTGCCGA
PEPC6	AK073703	F: ATGTCTGCCAGGCTTACACGAT R: CGGCTTAGACCAGTCCATGATC
PEPC7	AK242583	F: GAGTATTTCCGCCTTGCAACAC R: ACGGAGTGATTCAATGCCTCC
ACTIN	AK070531	F: GACAATGGAACCGGAATGGTC R: CCCAACCATAACGCCTGTATGT

the heading stage (Fig. 1). Results also revealed significant ($P < 0.05$) changes in the root length, plant height and dry weight under the no nitrogen level (0×N), low nitrogen level (0.1×N) and high nitrogen level (5×N) compared to the normal nitrogen level (1×N). At the tillering stage, compared to the 1×N level, there were 39.7% and 12.9% increases in the root length, 23.8% and 8.0% increases in the root dry weight under the 0×N and 0.1×N levels, respectively; there were 32.6% and 24.2% decreases in the shoot and plant dry weight under the 0×N level; while no significant ($P<0.05$) changes in the plant height were observed (Fig. 1). At the heading stage, compared to the 1×N level, there were 33.9% and 20.5% increases, 12.9% decrease in the root length, 63.9% and 47.2% decreases, 52.7% increase in the shoot dry weight, 62.1% and 48.0% decreases, 53.3% increase in the plant dry weight under the 0×N, 0.1×N and 5×N levels, respectively; there were 27.5% and 17.1% decreases in the plant height, 47.7% and 35.6% decreases in the root dry weight under the 0×N and 0.1×N levels, respectively (Fig. 1).

Physiological analysis of rice plant under four different nitrogen levels

As the cellular carbon and nitrogen metabolism was demonstrated to be tightly coordinated, we investigated the leaf SPAD value, stem nitrate concentration, the concentrations of soluble proteins and carbohydrates in the roots, stems and leaves at the tillering stage and heading stage, together with the leaf photosynthesis capacity, total carbon and nitrogen concentrations and carbon/nitrogen ratio in the roots, stems and leaves at the tillering stage to study the effect of different nitrogen levels on the carbon and nitrogen metabolic capacity in the rice plant under four different nitrogen levels (0×N, 0.1×N, 1×N and 5×N). Our results demonstrated that the leaf SPAD value, stem nitrate concentration, soluble proteins, photosynthetic rate, stomatal conductance and total nitrogen concentration increased with increasing nitrogen levels, whereas an opposite trend was observed for soluble carbohydrates and carbon/nitrogen ratio which decreased with increasing nitrogen levels (Figs 2, 3).

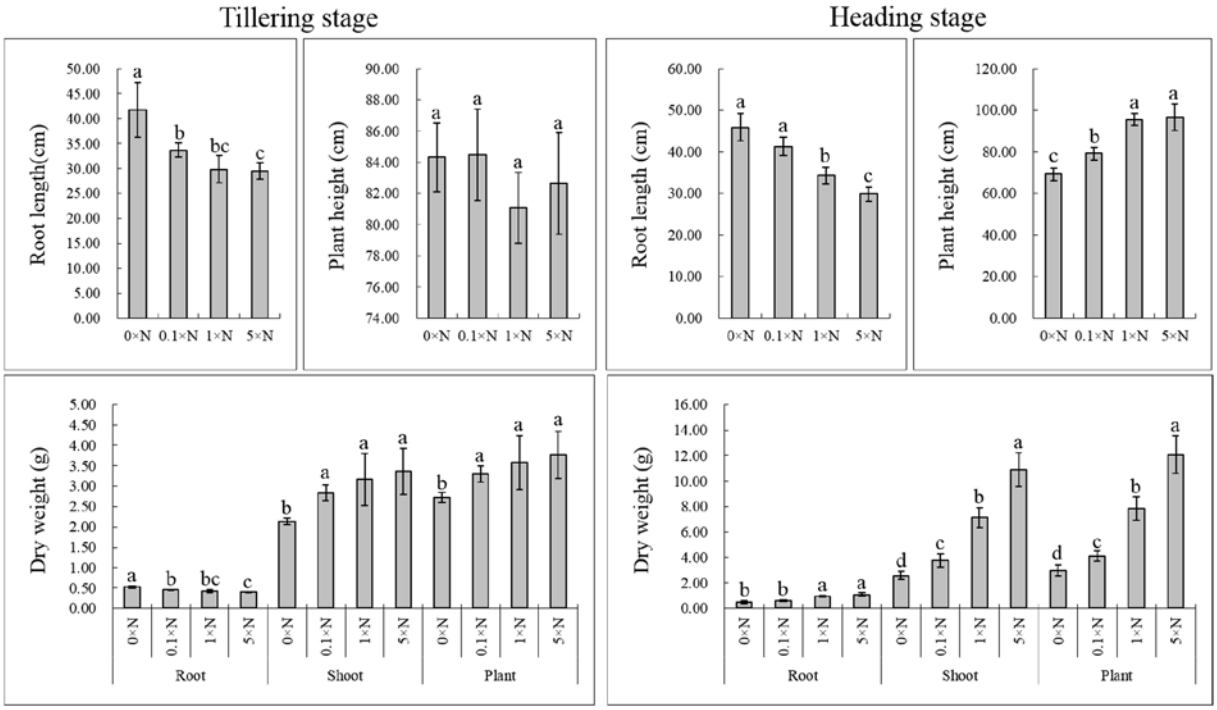


Fig. 1. The root length and plant height, the dry weight of the root, shoot and plant at the tillering stage and the heading stage under four different nitrogen levels (0×N, 0.1×N, 1×N and 5×N). Values are the mean ± SD of ten randomly selected plants. a, b, c, d indicate the significant differences among four different nitrogen levels at the level of $P = 0.05$.

Compared to the 1×N level, 21.3% and 6.7% decreases in the leaf SPAD value at the tillering stage, 30.5% and 13.6% decreases at the heading stage were observed under the 0×N and 0.1×N levels, respectively (Fig. 2). For the stem nitrate concentration, compared to the 1×N level, there were 82.3% and 51.2% decreases, 37.3% increase at the tillering stage, whereas 55.5% and 41.7% decreases, 23.7% increase at the heading stage under the 0×N, 0.1×N and 5×N levels, respectively (Fig. 2). Our results showed that most of the soluble proteins were in the leaves, whereas most of the soluble carbohydrates resided in the stems. For the soluble proteins at the tillering stage, compared to the 1×N level, 21.2% decrease and 50.9% increase in the roots, 30.5% decrease and 126.0% increase in the stems were displayed under the 0×N and 5×N levels, respectively; 31.5% and 36.1% decreases in the leaves were displayed under the 0×N and 0.1×N levels, respectively (Fig. 2). While at the heading stage, compared to the 1×N level, 46.1% and 42.8% decreases in the roots, 58.6% and 16.8% decreases in the stems, 45.7% and 36.1% decreases in the leaves were observed under the 0×N and 0.1×N levels, respectively; and 56.0% increase in the stem was also observed under the 5×N level (Fig. 2). For the soluble carbohydrates at the tillering stage, compared to the 1×N level, there were 65.8% and 45.3% increases in the roots under the 0×N and 0.1×N levels, respectively; there were 381.8% and 270.5% increases, 22.3% decrease in the stems under the 0×N, 0.1×N and 5×N levels, respectively; there were 34.2% and 78.8% increases in the leaves under the 0×N and 0.1×N levels, respectively (Fig. 2). While at the heading stage, compared to the 1×N level, there were 122.9% and 14.4% increases in the roots and leaves under the 0×N level, respectively; there were 149.9% and 66.2% increases, 69.8% decrease in the stems under the 0×N, 0.1×N and 5×N levels, respectively (Fig. 2).

For the photosynthesis capacity evaluation, we tested the photosynthetic rate, stomatal conductance, intercellular CO_2 concentration and transpiration rate in the flag leaf at the heading stage. There were no significant ($P < 0.05$) changes in the intercellular CO_2 concentration and transpiration rate under the 0×N, 0.1×N and 5×N levels compared to the 1×N level; while there was 34.2% decrease in the photosynthetic rate under the 0.1×N level, 31.7% and 29.3% decreases, 29.3% increase in the stomatal conductance under the 0×N, 0.1×N and 5×N levels, respectively (Fig. 3). Additionally, we analyzed the concentrations of the total carbon and nitrogen, carbon/nitrogen ratio in the roots, stems and leaves at the tillering stage to evaluate the carbon and nitrogen balance in the rice plants under four different nitrogen levels. Results showed that the treatments of different nitrogen levels caused larger variations in the total nitrogen concentrations than the total carbon concentrations which resulted significant changes in the carbon/nitrogen ratio. Compared to the 1×N level, 2.8% and 2.6% increases in the roots, 3.4% and 5.1% increases in the stems, 3.3% and 2.3% decreases in the leaves of the total carbon concentrations were observed under the 0×N and 0.1×N levels, respectively (Fig. 3). For the analysis of total nitrogen concentrations, compared to the 1×N level, there were 35.3% and 22.8% decreases in the roots under the 0×N

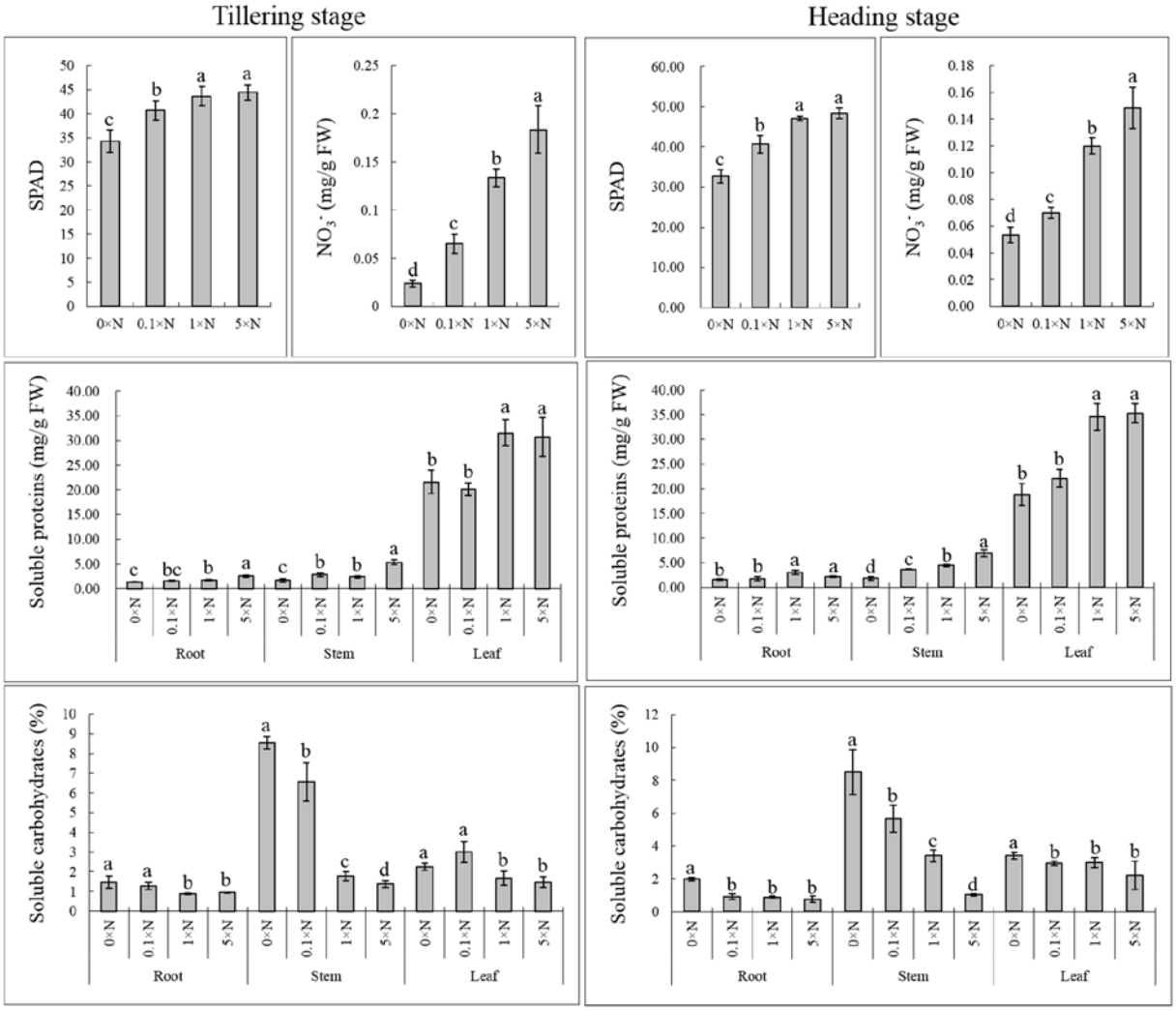


Fig. 2. The leaf SPAD value, stem nitrate concentration and the concentrations of soluble proteins and carbohydrates in the roots, stems and leaves at the tillering stage and the heading stage under four different nitrogen levels (0×N, 0.1×N, 1×N and 5×N). The SPAD values are mean ± SD of ten randomly selected plants. Other values are the mean ± SD from three biological replicates using three randomly mixed plant materials. a, b, c, d indicate the significant differences among four different nitrogen levels at the level of $P = 0.05$.

and 0.1×N levels, respectively; there were 60.7% and 42.6% decreases, 24.6% increase in the stems, 43.0% and 22.4% decreases, 6.1% increase in the leaves under the 0×N, 0.1×N and 5×N levels, respectively (Fig. 3). Oppositely, for the carbon/nitrogen ratio, compared to the 1×N level, there were 58.7% and 33.2% increases in the roots under the 0×N and 0.1×N levels, respectively; there were 161.9% and 83.0% increases, 20.0% decrease in the stems, 69.3% and 26.2% increases, 5.8% decrease in the leaves under the 0×N, 0.1×N and 5×N levels, respectively (Fig. 3).

Metabolic profiling analysis under the low nitrogen and normal nitrogen levels

To study the effect of nitrogen on the individual metabolites involved in the carbon and nitrogen metabolic pathways, we analyzed the sugars, organic acids and free amino acids individually in the roots and leaves of rice plants at the tillering stage under the low nitrogen (0.1×N) and normal nitrogen (1×N) levels. Figure 4 displays the fold change that corresponds to the ratio of 0.1×N treatment/1×N treatment calculated by the concentration of these individual metabolites. In the leaves, the low nitrogen treatment caused visible decreases in the concentrations of total sugars (<0.08 fold) and total organic acids (<0.68 fold). Compared to the 1×N level, dramatic decreases in fructose (<0.12 fold), xylitol (<0.002 fold), glutaric acid (<0.19 fold), pyruvate (<0.11 fold), succinate (<0.002 fold), aconitase (<0.16 fold), proline (<0.13 fold) and phenylalanine (<0.16 fold) were observed in the leaves under the 0.1×N level (Fig. 4). Additionally, several free amino acids increased dramatically, such as glutamine (>5.90 fold), threonine (>17.89 fold), alanine (>72.09 fold) and valine (>19.75 fold) (Fig. 4). In the roots, the low nitrogen treatment caused visible increases in the concentrations of total sugars (>2.05 fold), total organic acids (>1.45 fold) and total free amino acids (>1.65 fold). Compared to the 1×N level, dramatic increases in ascorbic acid (>16.00

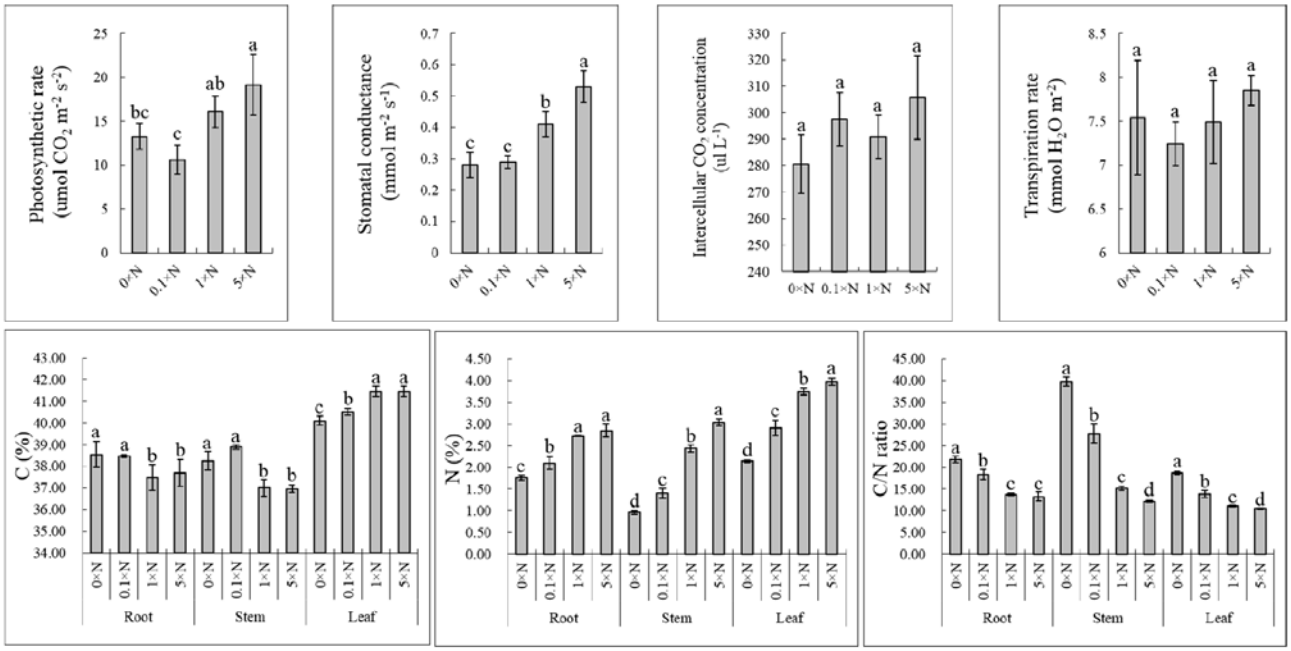


Fig. 3. The leaf photosynthesis parameters, the concentrations of carbon and nitrogen, the carbon/nitrogen ratio in the roots, stems and leaves at the tillering stage under four different nitrogen levels (0×N, 0.1×N, 1×N and 5×N). The values of photosynthesis parameters are mean ± SD of ten randomly selected plants. Other values are the mean ± SD from three biological replicates using three randomly mixed plant materials. a, b, c, d indicate the significant differences among four different nitrogen levels at the level of $P = 0.05$.

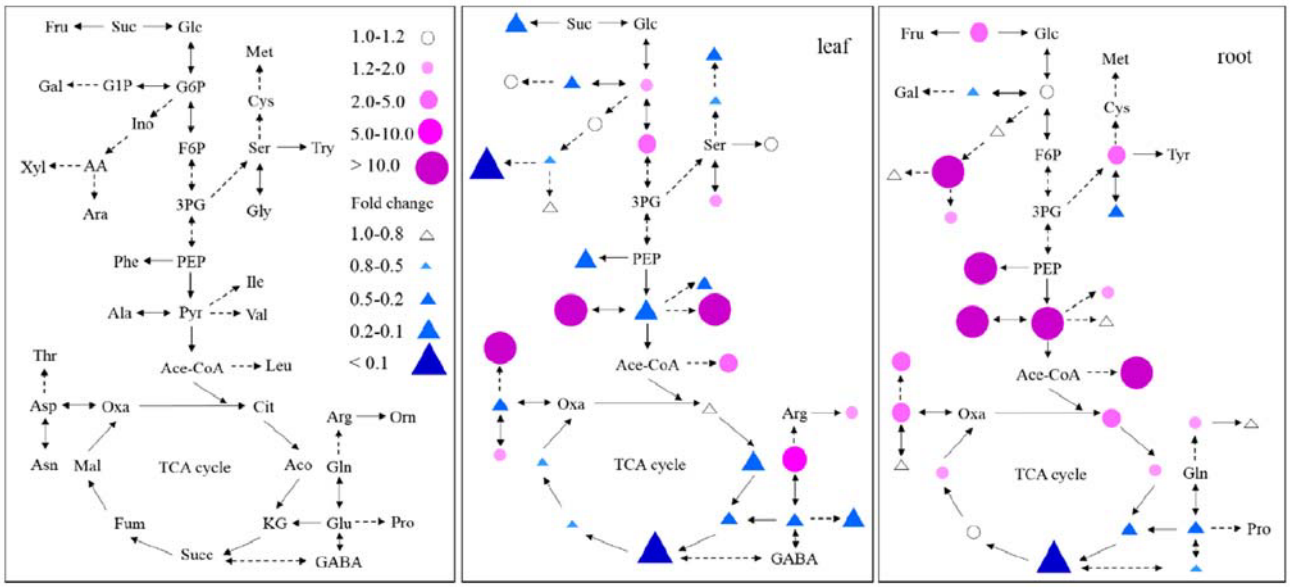


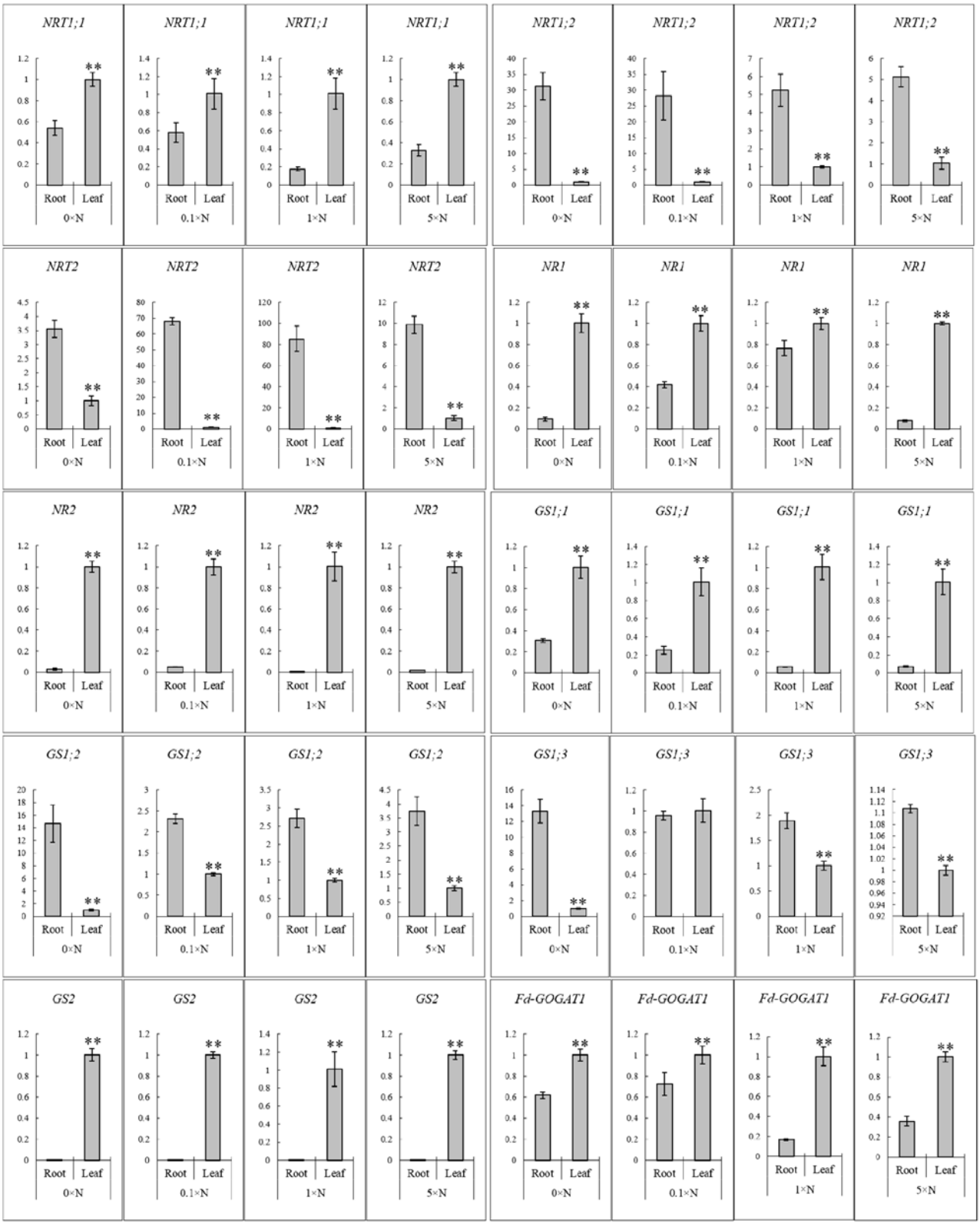
Fig. 4. The fold change corresponds to the ratio of the concentration of individual metabolites involved in carbon and nitrogen metabolism in the roots and leaves under the low nitrogen level (0.1×N) relative to the normal nitrogen level (1×N) at the tillering stage. Glc, glucose; Suc, sucrose; Fru, Fructose; F6P, Frutose-6-P; G6P, Glucose-6-P; G1P, Glucose-1-P; Gal, galactose; Ino, Inositol; AA, Ascorbic acid; Ara, Arabinose; Xyl, Xylitol; 3PG, 3-P-glycerate; PEP, P-enolpyruvate; Pyr, Pyruvate; Ace-CoA, acetyl-CoA; Cit, Citrate; Aco, Aconitase; KG, Ketoglutarate; Succ, Succinate; Fum, Fumarate; Mal, Malate; Oxa, oxaloacetate; Glu, Glutamate; Gln, Glutamine; Arg, Arginine; Pro, Proline; Orn, Ornithine; GABA, Aminobutyric; Asp, Aspartate; Asn, Asparagine; Ile, Isoleucine; Met, Methionine; Thr, Threonine; Ala, Alanine; Val, Valine; Leu, Leucine; Phe, Phenylalanine; Try, Tryptophan; Ser, Serine; Gly, Glycine; Cys, Cysteine. Red dots indicate increased metabolites and blue triangles indicate decreased metabolites.

fold), pyruvate (>691.29 fold), alanine (>253.60 fold), leucine (>949.52 fold) and phenylalanine (>19.83 fold) were observed; while the succinate (<0.04 fold) decreased dramatically in the roots under the 0.1×N level (Fig. 4).

Gene expression patterns under four different nitrogen levels

To analyze the effect of different nitrogen levels on the root and leaf expression patterns of key genes involved in carbon and nitrogen metabolism, the expression lev-

els of 20 genes encoding NRT (nitrate transporter), NR (nitrate reductase), GS (glutamine synthetase), GOGAT (glutamate synthase), RUBISCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) and PEPC (phosphoenolpyruvate carboxylase) in the roots and leaves at the tillering stage under four different nitrogen levels (0×N, 0.1×N, 1×N and 5×N) were analyzed by q-RT PCR. Figure 5 displays the relative expression levels of these genes in the roots and leaves. Results showed that 3 genes (*NRT1;2*, *NRT2* and *GS1;2*) mainly expressed in the roots and 12 genes (*NRT1;1*, *NR1*, *NR2*, *GS1;1*, *GS2*, *Fd-GOGAT1*, *Fd-GOGAT2*, *RU-*



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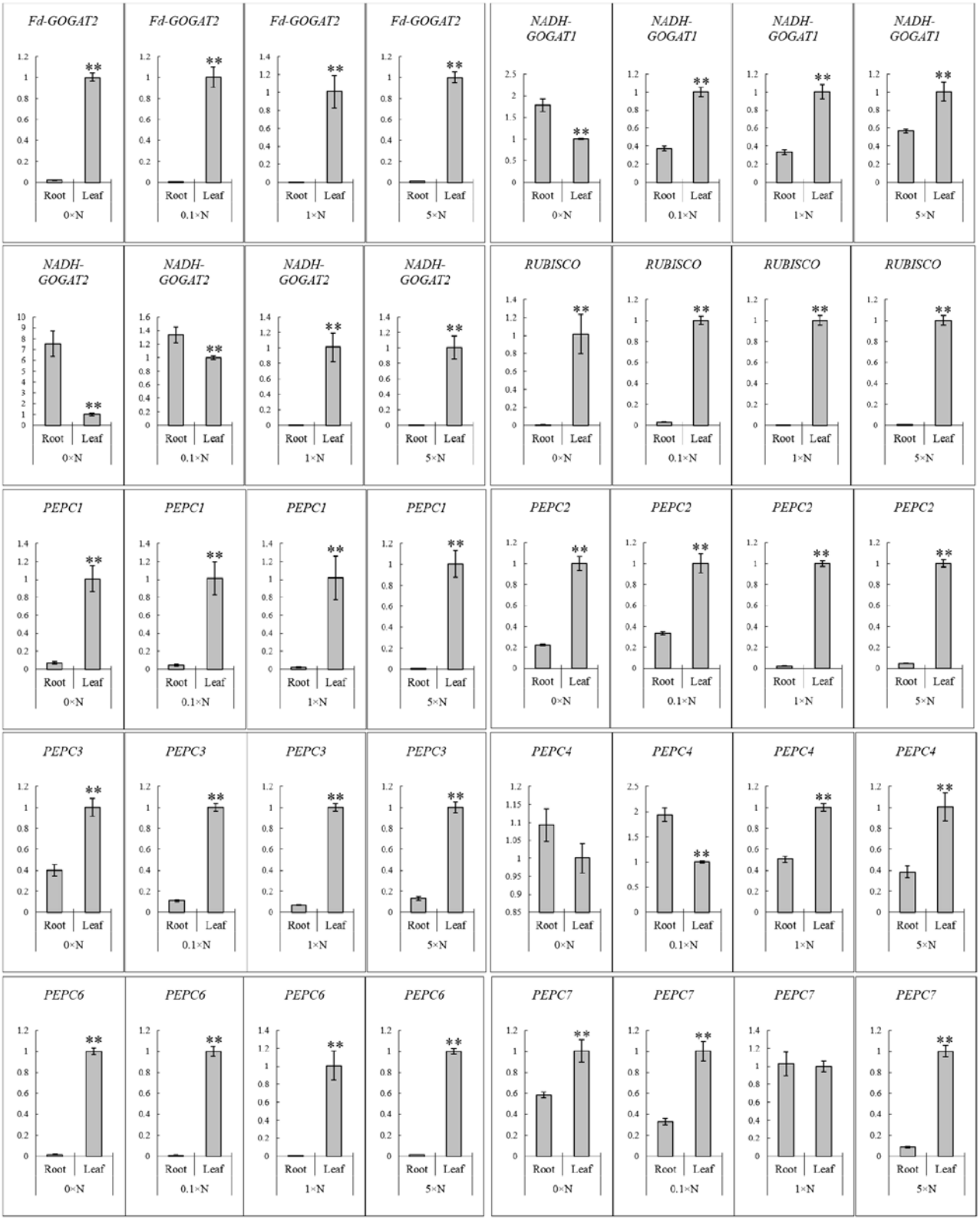

Fig. 5. The relative expression levels of the key genes involved in the carbon and nitrogen metabolism in the roots and leaves at the tillering stage under four different nitrogen levels (0×N, 0.1×N, 1×N and 5×N). NRT, nitrate transporter; NR, nitrate reductase; GS, glutamine synthetase; GOGAT, glutamate synthase; RUBISCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; PEPC, phosphoenolpyruvate carboxylase. ** indicates the significant differences at the level of $P = 0.01$.

BISCO, *PEPC1*, *PEPC2*, *PEPC3* and *PEPC6*) mainly expressed in the leaves under four different nitrogen levels (Fig. 5). The expression patterns of the remaining 5 genes (*GS1;3*, *NADH-GOGAT1*, *NADH-GOGAT2*,

PEPC4 and *PEPC7*) altered under four different nitrogen levels (Fig. 5). For example, the expression levels of *GS1;3* in the roots were significantly ($P < 0.01$) higher than in the leaves under the 0×N, 1×N and 5×N levels, while there was no significant difference between the roots and leaves under the 0.1×N level (Fig. 5). The expression level of *NADH-GOGAT1* in the roots was significantly ($P < 0.01$) higher than in the leaves under the 0×N level, while the opposite results displayed under the 0.1×N, 1×N and 5×N levels (Fig. 5). The expression level of *NADH-GOGAT2* in the roots was significantly ($P < 0.01$) higher than in the leaves under the 0×N and 0.1×N levels, while the opposite results displayed under the 1×N and 5×N levels (Fig. 5). The expression pattern of *PEPC4* showed more complex that there was no significant difference of the *PEPC4* expression level between the roots and leaves under the 0×N level, while the *PEPC4* expression level was significantly ($P < 0.01$) higher in the roots than leaves under the 0.1×N level, and the opposite results displayed under the 1×N and 5×N levels (Fig. 5). The expression levels of *PEPC7* were significantly ($P < 0.01$) higher in the roots than leaves except that there was no significant difference between the roots and leaves under the 1×N level (Fig. 5).

Discussion

The effect of nitrogen level on plant growth

As plants are immovable, they are highly sensitive to the environment and respond accordingly for growth and survival (Mohd-Radzman et al. 2013). N is particularly important, because it provides the building blocks for protein production in plants and dictates crop yield (Mohd-Radzman et al. 2013). Understanding how rice grow and develop under diverse environmental nitrogen levels is crucial for improving rice productivity. In our study, to test the effect of different nitrogen levels on the rice growth, we analyzed the root length, plant height and dry weight under four different nitrogen levels (0×N, 0.1×N, 1×N and 5×N). Results showed that the plant height and dry weight increased with increasing nitrogen levels, whereas an opposite trend was observed for the root length, which decreased with increasing nitrogen levels. Similarly, the sunflower plants grown with 20 mM nitrate showed greater leaf area and specific leaf mass than the plants grown with 2 mM nitrate (Agüera et al. 2010). Hodge et al. reported that N levels strongly influenced root architecture and crop yields (Hodge 2006; Garnett et al. 2009). Generally, high nitrate (≥10 mM) imparts systemic inhibition of lateral and primary root growth whereas low nitrate (≤1 mM) promotes both (Robinson et al. 1999; Zhang et al. 1999; Walch-Liu et al. 2006; Ruffel et al. 2011).

The effect of nitrogen level on plant physiological status

To study the possible effect of environmental N level on carbon-nitrogen metabolism in rice, we have analyzed the leaf SPAD value and photosynthesis capacity, stem nitrate concentration, the concentrations of soluble proteins and carbohydrates, total carbon and nitrogen concentrations, carbon/nitrogen ratio in the root, stem and leaf tissues under four different nitrogen levels (0×N, 0.1×N, 1×N and 5×N). Our results demonstrated that the leaf SPAD value, stem nitrate concentration, soluble proteins, photosynthetic rate, stomatal conductance and total nitrogen concentration increased with increasing nitrogen levels, whereas an opposite trend was observed for soluble carbohydrates and carbon/nitrogen ratio which decreased with increasing nitrogen levels. These results indicated high environmental N level promoted the N transport and assimilation capacity, together with the consumption of carbon skeletons. Generally, plants grown in high N are often taller, with more tillers and greater leaf area, which requires more carbohydrates to constitute the plant structures; while the low N limit plant growth and development couples with the accumulation of carbohydrates (Yoshida & Ahn 1968; Hirano et al. 2005). In addition, the transformation of inorganic nitrogen absorbed by roots into organic nitrogen compounds requires carbon skeleton produced by photosynthesis and consequently causes a decrease in carbohydrates accumulation (Gebbing et al. 1999). Similar results were also reported that the sunflower plants grown with high nitrate presented more total protein and chlorophyll content than leaves of plants grown with low nitrate (Agüera et al. 2010). The concentration and total mass of stem carbohydrates in rice plants were larger under low N condition compared with high N condition (Pan et al. 2011).

The effect of low nitrogen level on individual carbon-nitrogen metabolites

Besides, we analyzed the sugars, organic acids and free amino acids individually in the roots and leaves of rice plants at the tillering stage under the low nitrogen (0.1×N) and normal nitrogen (1×N) levels to study the effect of environmental N level on the individual metabolites involved in the carbon and nitrogen metabolic pathways. Results showed the individual metabolites changed significantly in rice plants under low N condition when compared to the normal N condition. Nitrogen deficiency often results in sugar accumulation (Wingler et al. 1998, 2006; Masclaux-Daubresse et al. 2007). The soluble sugar content of rice and wheat plants grown under high nitrogen conditions were lower than those grown under low nitrogen conditions (Yoshida & Ahn 1968; Gebbing & Schnyde 1999; Hirano et al. 2005; Ruuska et al. 2008; Scofield et al. 2009). Interestingly, our results showed the low nitrogen treatment caused a visible accumulation (>2.05 fold) in the concentration of total sugars in roots, while a significant decrease (<0.08 fold) in leaves. Additionally, the low nitrogen treatment caused a visible decrease in the concentration total organic acids (<0.68 fold) in the leaves, while visible increases in the concentrations of total organic acids (>1.45 fold) and total free amino acids (>1.65 fold) in the roots. Various changes were observed in the individual free amino acids by the low N treatment, such as proline, glutamine, threonine, leucine,

alanine and valine. It is reasonable that nitrogen deficiency always reduce the protein synthesis and cause protein degradation in plants. During protein degradation, free amino acids are released and probably interconverted, hydrolyzed, catabolized or exported without modifications to another place in the plant (Diaz et al. 2005).

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