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RESEARCH

Effects of elevated nitrogen application on nitrogen partitioning, plant growth, grain quality and key genes involved in glutamate biosynthesis among three rice genotypes

Jun Wang[1], Yi-peng Lu[2], Jie Wang[2], Rui-xin Xu[2], Jun Li[4], Wei Hu[2], Ya-jun Xiong[2], Yun-bo Zhang[3], Xiao-yan Wang[3], and Xiao-hai Tian[3*]

[1]Yangtze University, College of Agriculture, Jingzhou, 434025, Hubei, P.R. China.
[2]Yangtze University, Engineering Research Center of Ecology and Agricultural Use of Wetland, Jingzhou 434023, Hubei, P.R. China.
[3]Yangtze University, Hubei Collaborative Innovation Center for Grain Industry, Jingzhou 434023, Hubei, P.R. China.
[*]Corresponding author (xiaohait@sina.com).
[4]Guangxi University, College of Agronomy, Nanning, 530005, Guangxi, P.R. China.

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ABSTRACT

The N absorption and assimilation is critical for the rice (*Oryza sativa* L.) yield increase when overdose N was applied in rice production. Three different rice genotypes, 'Quanliangyou 1' (Q1), 'Quanliangyou 681' (Q681) and 'Huanghuazhan' (HHZ), were selected to investigate the effects of elevated N input on the N partitioning, plant growth, grain quality and key genes involved in glutamate biosynthesis. Under increasing N inputs (0, 120, 180, 250 kg ha^{-1}), N content in leaf, culm, seed and root were increased significantly. The increased N was preferentially deposited in leaf and culm. Tiller number, panicle number and length were also proved to be significantly promoted, but plant height and 1000-grain weight were nonsignificantly affected under elevated N input. Under high N input, seed protein content was elevated, while fatty acid and amylose content remained unchanged in comparison to low N input, but amylopectin content decreased. For the key genes in N assimilation, glutamine synthetase (*OsGS1;1*) could be induced by increasing N input (0 to 180 kg ha^{-1}) but higher N input (250 kg ha^{-1}) inhibit its expression, which showed similar response pattern with the glutamine synthetase activity. Although different rice genotypes showed similar response pattern to elevated N input, each genotype varied a lot in certain phenotypic indexes. And the response pattern of all these phenotypic characteristics to elevated N input was independent of rice genotype. These findings suggest that elevated N input could promote rice growth, reallocate N content in different tissues, and have negative impact on grain quality. This study provided physiological and molecular foundation for rice breeding and cultivation under high N input.

Key words: Grain quality, growth promotion, nitrogen input, nitrogen partitioning, *Oryza sativa*, rice.

INTRODUCTION

With the increasing human population globally, the demand of crop yield accelerated dramatically in last few decades. One of the common practices for higher crop yield is the application of over-dose fertilizer. Of the three basic nutrients (N, P, and K) for plant growth and development, N is believed to be the most essential nutrient for plant growth and a major restriction factor in plant productivity (Tabuchi et al., 2007). Cereal yield and N fertilizer have been highly correlated in a linear way during the past 40 yr (Smil, 2001; Cassman et al., 2002; Ladha et al., 2016). Statistics showed that the N

fertilizer consumption has augmented for approximately 17-fold in the last four decades (Ali et al., 1999; Dobermann and Cassman, 2004; Rahn et al., 2009). However, N nutrient in soil has not been fully applied for crop growth and development, and finally converted into crop yield. It is estimated that only 50% or less N fertilizer applied has been used for aboveground biomass production in cereals, and the rest has been wasted by dissipating into environment via surface runoff, leaching, volatilization, and denitrification, which have resulted in severe environmental disasters (Giles, 2005; Nestler et al., 2011; Simplicio et al., 2016). Therefore, how to achieve efficient use of N via cultivation techniques and fertilizer management is of great concern for the agronomist focusing on crop cultivation.

N fertilization significantly increases tissue N concentration (Ye et al., 2014). It was reported that approximate 70%-90% of the plant total N come from the remobilized N from vegetative organs (Mae, 1997). Nitrogen partitioning within plant contributes to the growth dynamics, portioning of biomass, and the grain quality. Due to the complexity caused by inherent factors, including 1000-grain weight, grain number per panicle and productive culms in addition to plant N management, studies focused on the N use efficiency among different rice genotypes are limited (Samonte et al., 2006). It was reported that the increase of N in leaves could increase the hull size and grain filling, whilst the number of degenerate spikelet decreased (Singh and Verma, 2013).

The critical step restricting the N use efficiency is the capability of plant in acquiring N from applied fertilizer (Shrawat et al., 2008). Urea, as a form of N fertilizer with lower application cost than other nitrogenous sources, is the major N carrier worldwide in crop production (Fageria et al., 2011). The assimilation of urea N (NH_4) was achieved by a coupled reaction mediated by glutamine synthetase (GS) and glutamate synthase (GOGAT). In rice, there are two types of GS, cytosolic and chloroplastic. Cytosolic GS is encoded by three genes (*OsGS1;1*, *OsGS1;2* and *OsGS1;3*), and chloroplastic GS is encoded by GS2 (*OsGS2*) (Tabuchi et al., 2007). *OsGS1;1*, *OsGS1;2*, *OsGS1;3* and *OsGS2* showed tissue specific expression pattern, which are abundant in shoot, root, spikelets, and leaf respectively (Tabuchi et al., 2005). For the GOGAT, there were three genes encoding either ferredoxin (Fd)-*GOGAT*, *NADH-GOGAT1*, or *NADH-GOGAT2*, of which *OsNADH-GOGAT2* is a newly identified gene expressed preferentially in fully expanded leaf blades and leaf sheaths (Tamura et al., 2011). The expression pattern could to some extent reflect the ammonium assimilation status of plant.

Unveiling of the response pattern of crops to large regime of N supply would be especially essential for better N management in crops. The objective of this study was to gain a better understanding of the effects on N partitioning, plant growth, grain quality, and expression pattern of key genes involved in glutamate synthesis among three rice genotypes under elevated N input, and provide essential information for better N management in cultivation.

MATERIALS AND METHODS

Plant materials and treatments

Two rice cultivars (*Oryza sativa* L. subsp. *indica* Kato), Quanliangyou 1 and Quanliangyou 681, were provided by Hubei All-win High-tech Seed Co., Ltd., and 'Huanghuazhan' was provided by Huazhong Agricultural University. Experiments were performed by orthogonal design, in which three different cultivars and four distinct N input (0, 120, 180, and 250 kg ha^{-1}), namely control, low N, moderate N and high N, and each treatment were supplied with basal fertilization of 122.5 kg P ha^{-1} and 150 kg K ha^{-1}. For each treatment, and increasing N doses were 0, 120, 180, and 250 kg ha^{-1}. Different cultivars and different N input were used as two factors. Rice seedlings were transplanted to soil filled pot after germination, and three plants in each pot were retained. Three pots were planted as technical replicates and each treatment was repeated for three times as biological replicates within the same growing season. All experiments in this study were carried out in the open-air conditions similar to field test, during the time period from 2 May to 30 August 2015 at Yangtze University, China.

Phenotypic analysis

During the plant growth, we characterized the growth period, including tillering date, heading date, booting date, flowering date, maturation date, plant height, effective tiller number and length, leaf area, panicle number and length, and leaf chlorophyll content, were characterized at heading stage, leaf weight, culm weight were determined after maturation. Flag leaf area (FLA) was calculated by L (leaf length) × W (width) in cm^2 as described by Wang et al. (2012). Leaf chlorophyll content was determined by SPAD-502 meter (Ling et al., 2011).

Glutamine synthetase activity

Raw protein containing glutamine synthetase (GS) was extracted from leaf tissue by the extraction buffer (2 mmol Mg^{2+} L^{-1}, 2 mmol dithiothreitol L^{-1}, 0.4 mol sucrose L^{-1}), then protein content was determined by Bradford protein assay (Bradford, 1976).

The GS activity was determined by measuring the absorbance of γ-glutamyl hydroxamate (GH) and ferric chloride formed complex at 540 nm with modifications (Hakeem et al., 2011). Firstly, the raw enzyme extract was incubated with reaction A (80 mmol Mg^{2+} L^{-1}, 20 mmol sodium glutamate L^{-1}, 20 mmol cysteine L^{-1}, 2 mmol EGTA L^{-1}, 80 mmol hydroxylamine hydrochloride L^{-1}, pH 7.4) and 0.7 mL adenosine triphosphate (ATP, 40 mmol L^{-1}) for 30 min, then 0.37 mol L^{-1} ferric chloride containing 0.2 mol L^{-1} trichloroacetic acid and 0.6 mol L^{-1} hydrochloric acid was added to mixture to display color for measurement. The absorbance was determined by automatic microplate reader (BioTek, Synergy 2). Enzyme activity was calculated by GS activity = A/(P V T), where A, P, V, and T represents micromoles of γ-GH, protein content, volume of raw enzyme extract used, and reaction time.

Nitrogen content and grain quality traits measurement

Different tissues of plant under different N supply conditions, namely root, stem, leaf, and seeds, were harvested at maturation stage for N content determination. Total N content of each tissue was determined by standard macro-Kjeldahl procedure (Horneck and Miller, 1998) using Kjeldahl apparatus (Ketuo KDY-9820, China). Brown rice of each treatment was made by brown rice machine (THU35-C, Satake, Penrith, New South Wales, Australia), and then grain quality related traits, i.e. protein content, fatty acid content, amylose and amylopectin content, and quality index were determined by standard program of near infrared spectrometer (DA7200, Perten Instruments, Hägersten, Sweden).

RNA extraction and real time PCR

Flag leaves of each plant in each pot at heading stage were harvested for RNA extraction. Total RNA was extracted from 100 mg leaf powder by RNAiso Plus (TaKaRa Code: D9108A). RNA was then treated by Recombinant DNase I (Ambion, AM2235, Life Technologies, Carlsbad, California, USA) to eliminate genomic DNA contamination. Approximately 2 μg DNase-treated RNA was then reverse transcribed into first strand (Takara, Code: D6110A) according to the user's manual.

Amplification of real-time PCR products was detected with a QuantStudio qPCR Detection System in a reaction mixture of 10 μL: 1 μL cDNA first strand product, 1 μL gene specific primer, 3 μL MiliQ water, and 5 μL SYBR Premix ExTaq II (Takara, RR82LR). The real time PCR was performed by the following programs: template denatured at 95 °C for 30 s, then followed by 40 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 5 s, after that 30 cycles at 60 °C were performed to obtain a melting curve. Geometric mean of expression level of *Actin* (AK100267), *Ubiquitin* (AK101547), and *Gapdh* (AK064960) were used as internal standard (Moraes et al., 2015). The relative expression level of *OsGS1;1* and *OsNADH-GOGAT2* were calculated against internal standard by 2 (-Delta Delta C(T)) method as described previously (Livak and Schmittgen, 2001). Gene specific primer targeted to *OsGS1;1*(LOC_Os02g50240.1) and *OsNADH-GOGAT2*(LOC_Os05g48200.1) could be found in Table 1.

Table 1. Primer sequence designed for qRT-PCR.

Primer name	Sequence(5'-3')
OsGOGAT2F	CCTGTCGAAGGATGATGAAGGTGAAACC
OsGOGAT2R	TGCATGGCCCTACTATCTTCGCATCA
OsGS1;1F	CAAGTCTTTTGGGCGTGATATTGTTGAC
OsGS1;1R	CACCTGATCACCGGCAGAAATGCCGACA
GAPDH1F	AAGCCAGCATCCTATGATCAGATT
GAPDH1R	CGTAACCCAGAATACCCTTGAGTTT
UBQ10-1F	TGGTCAGTAATCAGCCAGTTTGG
UBQ10-1R	GCACCACAAATACTTGACGAACAG
ACT11-1F	CAGCCACACTGTCCCCATCTA
ACT11-1R	AGCAAGGTCGAGACGAAGGA

Data analysis

All experiments of this study were based on the orthogonal design described in plant materials and treatment. Mean value of three individual plants of each pot were deemed as one individual replicate of each treatment. Differences among treatments and different cultivars were analyzed by One-way or Two-way ANOVA with significant level of $p < 0.05$ or $p < 0.01$ according to fixed model of Duncan's shortest significant range-test.

RESULTS

N partitioning in response to elevated N input

Clarification of the processes governing N fluxes, especially N assimilation and allocation in plant is of great significance to both environmental concerns and the quality of crop products (Ata-Ul-Karim et al., 2017). We studied the N partitioning among root, culm, leaf and seed at maturation stage as this stage could reflect the final N accumulation status. Under moderate N input (180 kg ha^{-1}), N contents were most abundant in seed (0.91 ± 0.02%), then followed by leaf (0.73 ± 0.03%), root (0.44 ± 0.03%) and culm (0.34 ± 0.01%) (Figure 1A). Compared with control (0 kg N ha^{-1}), low and moderate N input did not change N contents in root, culm, seed, and leaf. However high N input (250 kg ha^{-1}) significantly increased the N content in all these tissues (Figure 1B), indicating that higher N input exceeding 180 kg ha^{-1} could enhance overall N content of rice plant. Compared with N content under moderate N input (180 kg ha^{-1}), higher N dose (250 kg ha^{-1}) increased N content by 29.55%, 58.82% 31.87%, and 67.12% in root, culm, seed and leaf respectively (Figure 1B), revealing that high N supply had stronger impact on above-ground vegetative organs than on reproductive organs and under-ground organs. Correlation analysis showed that seed N content was highly correlated to N content in culm and leaf (Pearson correlation coefficient $r > 0.84$, $p < 0.01$, Table 2), but less correlated to N content in root (Pearson correlation coefficient $r = 0.754$, $p < 0.01$, Table 2).

Impact of elevated N input on plant growth

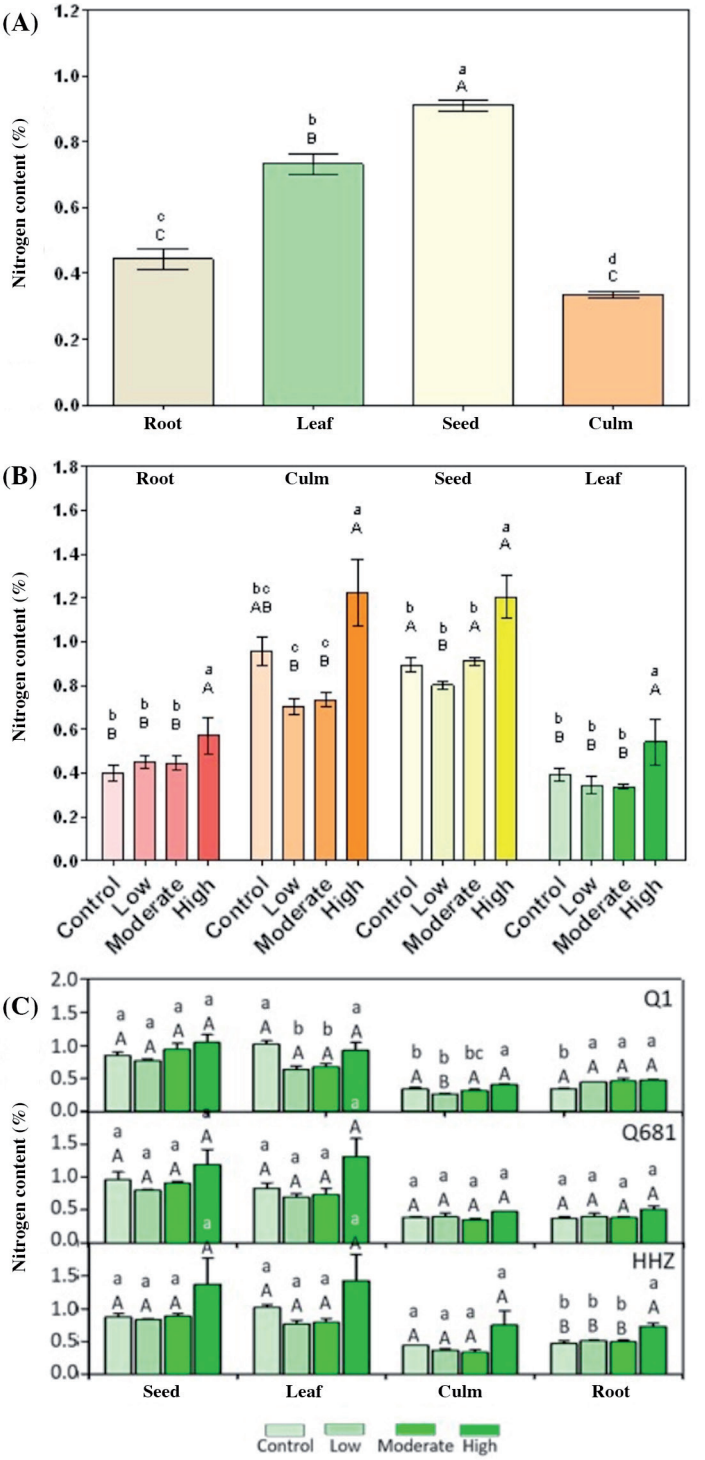
Since N is an essential element for plant growth, the vegetative and productive growths among three distinct rice genotypes (Q1, Q681, and HHZ) under different N input (0, 120, 180, and 250 kg ha^{-1}) were investigated in our study. The plant height without extra N input (control) was 65.22 ± 0.58 cm on average, and increased by 32.6% and 45.7% respectively when the N input was 120 and 180 kg ha^{-1}, but under 250 kg ha^{-1} the plant height was nonsignificantly different from that under 180 kg ha^{-1} (Figure 2A), indicated 180 kg ha^{-1} N was possibly the optimal N input for plant height. However, the average effective tiller number kept climbing from 7.78 ± 0.11 per plant to 27.78 ± 2.30 per plant as the N input increased from 0 to 250 kg ha^{-1} (Figure 2B). Similar trend was observed in panicle number, leaf weight, and culm weight as well (Figures 2C, 2D, 2E). Correlation analysis showed that tiller number, leaf number, stem number, leaf weight, and culm weight were highly correlated to each other (Pearson correlation coefficient $r > 0.8$, $p < 0.01$, Table 2). These results indicated that tillering was more sensitive than plant height in response to high N input.

Elevated N input enhanced leaf area, leaf chlorophyll content, panicle length, and 1000-grain weight

Leaf is an important trait in cereal crops, which serves as the main source of photosynthetic products for primary sink (Wang et al., 2012). Flag leaves were reported to be associated with improved 1000-grain weight and panicle length in rice (Lee et al., 2017). In this study, leaf area, leaf chlorophyll content of flag leaves, 1000-grain weight and panicle length from each rice genotype under different N input were characterized. Leaf area of flag leaves was measured at heading stage. Under 0 kg ha^{-1} N input the leaf area was 21.25 ± 1.06 cm^2 and significantly increased to 57.68 ± 7.24 cm^2 ($p < 0.01$) as the N supply increased to 250 kg ha^{-1} (Figure 3A). Leaf chlorophyll content (SPAD) of flag leaves without N input (Control: 0 kg ha^{-1}) was 30.91 ± 0.63 on average, which significantly increased by 19.9% to 37.05 ± 1.17 under 180 kg ha^{-1} ($p < 0.01$, Figure 3B). However, the leaf chlorophyll content under N input of 250 kg ha^{-1} only increased by 4.99% compared with that under 180 kg ha^{-1} ($p < 0.05$), suggested the maximum leaf chlorophyll content could be expected within small N input regime around 250 kg ha^{-1}.

To better understand the impact of high N input on yield, panicle length and 1000-grain weight was studied among these three rice genotypes. Under 0 and 120 kg ha^{-1} N input, the panicle length was almost the same (22.95 ± 0.65 and 23.39 ± 0.45 cm, respectively), and significantly increased to 24.13 ± 0.51 and 25.20 ± 0.51 cm under 180 and 250 kg ha^{-1},

Figure 1. Nitrogen distribution in different tissues.



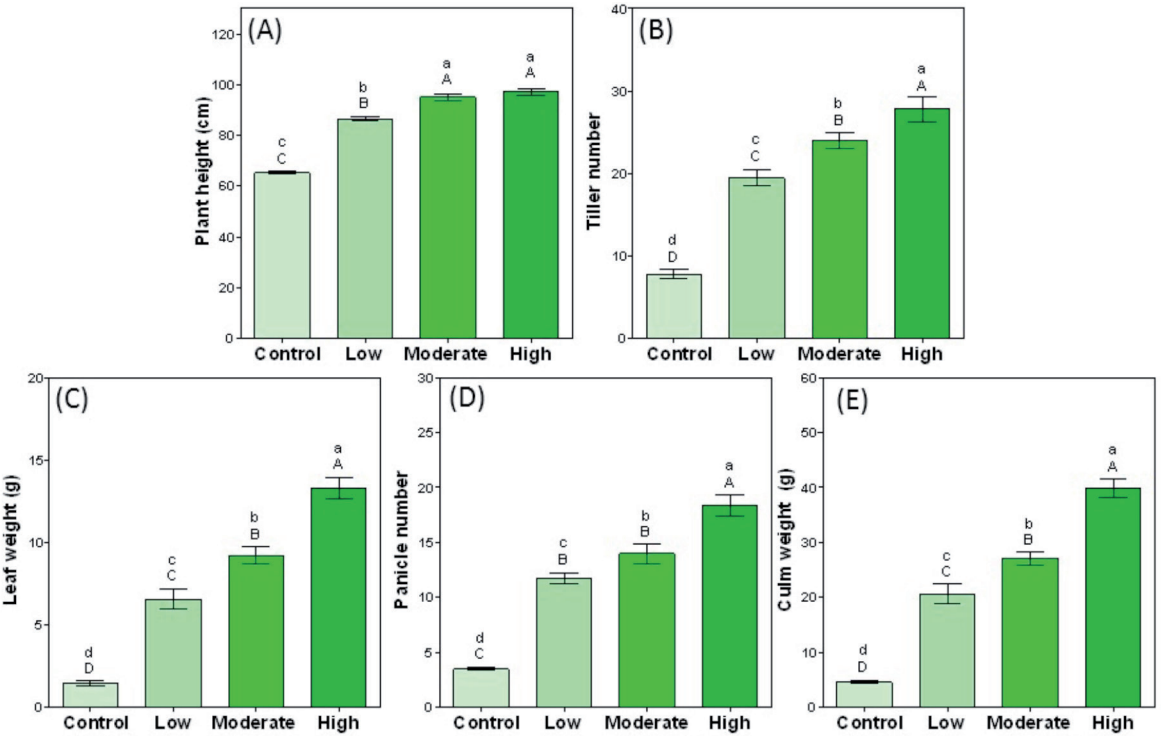
(A) Nitrogen content in different tissues (stem, seed, leaf and root) under moderate N dose (180 kg ha^{-1}), One-way ANOVA; (B) Nitrogen content in stem, seed, leaf and root under different N doses, Two-way ANOVA; (C) Nitrogen content in different tissues among Q1, Q681, and HHZ, One-way ANOVA.
Control: 0 kg N ha^{-1}; Low: 120 kg N ha^{-1}; Moderate: 180 kg N ha^{-1}; High: 250 kg N ha^{-1}. Q1: 'Quangliangyou 1', Q681: 'Quangliangyou 681', HHZ: 'Huanghuazhan'.
Significance of 0.05 and 0.01 probability level is indicated by lower-case and uppercase letters on top of error bar (standard error). These experiments were performed by three independent biological replicates.

Table 2. Correlation between different phenotypic indexes.

Indexes	Plant height	Leaf chlorophyll content	Tiller number	Spike number	Spike length	Leaf area	Leaves weight	Stem weight	1000-grain seed weight	N-content in root	N-content in leaf	N-content in seed	N-content in stem	Seed protein content	Quality index	Amylopectin content	Amylose content
	cm	%			cm	cm^2	g			%							
Leaf chlorophyll content, %	0.728																
	0.007																
Tiller number	0.932	0.824															
	0.000	0.001															
Spike number	0.904	0.773	0.973														
	0.000	0.003	0.000														
Spike length, cm	0.694	0.398	0.558	0.544													
	0.012	0.200	0.059	0.068													
Leaf area, cm^2	0.893	0.630	0.792	0.767	0.860												
	0.000	0.028	0.002	0.004	0.000												
Leaves weight, g	0.917	0.796	0.964	0.967	0.671	0.860											
	0.000	0.002	0.000	0.000	0.017	0.000											
Stem weight, g	0.901	0.822	0.972	0.965	0.665	0.853	0.985										
	0.000	0.001	0.000	0.000	0.018	0.000	0.000										
1000-grain seed weight, g	0.171	0.110	-0.057	-0.159	0.502	0.372	-0.033	-0.001									
	0.596	0.733	0.860	0.622	0.096	0.234	0.920	0.998									
N-content in root, %	0.470	0.405	0.662	0.722	0.202	0.323	0.677	0.665	-0.633								
	0.123	0.191	0.019	0.008	0.529	0.305	0.016	0.018	0.027								
N-content in leaf, %	0.021	0.346	0.268	0.324	0.209	0.104	0.379	0.395	-0.425	0.646							
	0.949	0.271	0.399	0.304	0.515	0.748	0.224	0.204	0.168	0.023							
N-content in seed, %	0.396	0.599	0.578	0.610	0.437	0.404	0.677	0.673	-0.239	0.754	0.846						
	0.203	0.040	0.049	0.035	0.156	0.193	0.016	0.017	0.455	0.005	0.001						
N-content in stem, %	0.165	0.442	0.394	0.467	0.066	0.067	0.446	0.445	-0.556	0.812	0.848	0.870					
	0.608	0.150	0.205	0.126	0.839	0.836	0.146	0.147	0.060	0.001	0.000	0.000					
Seed protein content, %	0.498	0.518	0.692	0.719	0.422	0.512	0.760	0.757	-0.429	0.787	0.740	0.812	0.668				
	0.100	0.084	0.013	0.008	0.172	0.089	0.004	0.004	0.164	0.002	0.006	0.001	0.018				
Quality index, %	-0.245	-0.228	-0.071	-0.011	0.140	-0.096	0.068	0.050	-0.447	0.482	0.752	0.580	0.528	0.605			
	0.443	0.476	0.827	0.974	0.664	0.767	0.834	0.878	0.145	0.112	0.005	0.048	0.077	0.037			
Amylopectin content, %	0.668	0.445	0.679	0.629	0.059	0.377	0.489	0.529	-0.109	0.333	-0.318	-0.085	-0.033	0.148	-0.530		
	0.018	0.147	0.015	0.028	0.854	0.227	0.107	0.077	0.735	0.290	0.313	0.793	0.920	0.647	0.076		
Amylose content, %	0.717	0.260	0.688	0.668	0.424	0.621	0.628	0.630	0.056	0.342	-0.241	0.068	-0.145	0.368	-0.133	0.700	
	0.009	0.415	0.013	0.018	0.169	0.031	0.029	0.028	0.862	0.276	0.451	0.834	0.652	0.239	0.680	0.011	
Fatty acid content, %	0.202	0.724	0.282	0.259	0.225	0.218	0.313	0.350	0.197	0.068	0.473	0.556	0.490	0.262	-0.068	-0.120	-0.385
	0.528	0.008	0.374	0.416	0.482	0.497	0.321	0.265	0.538	0.833	0.120	0.061	0.106	0.411	0.833	0.711	0.216

*Pearson correlation efficient larger than 0.80 were highlighted by yellow; **p value < 0.01 were indicated by red.

Figure 2. Alteration of plant growth related indexes in response to different N input.



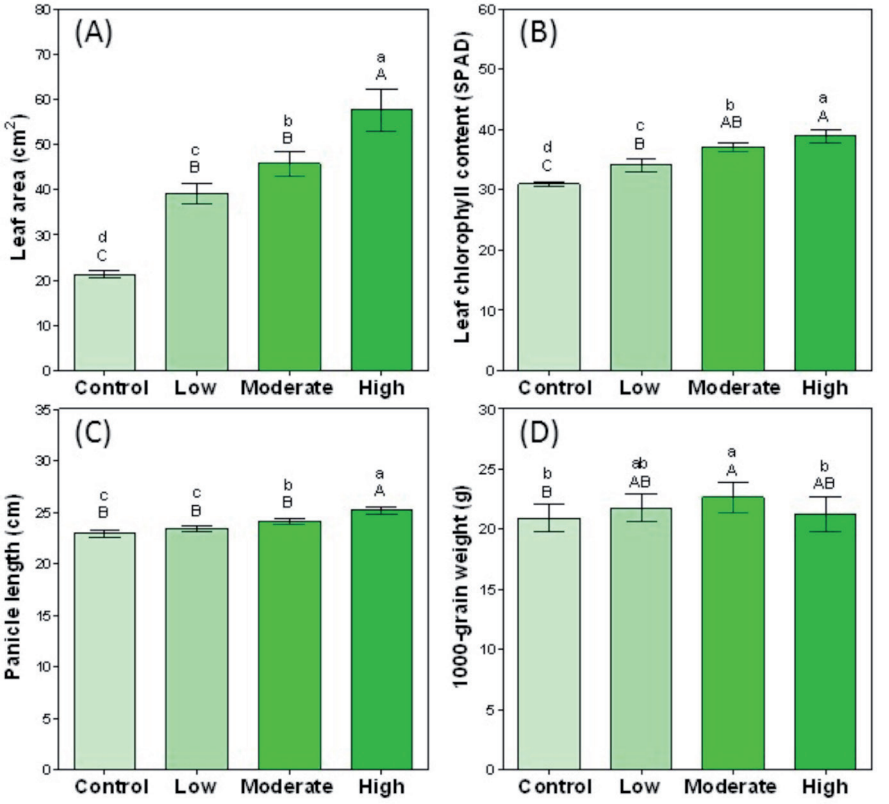
(A) - (E) Plant height (cm), tiller number, leaf weight (g), panicle number and culm weight (g).
Different N inputs are indicated by control (0 kg ha^{-1}), low (120 kg ha^{-1}), moderate (180 kg ha^{-1}), and high (250 kg ha^{-1}). Lower-case and uppercase letters on the top of error bar (standard error) represent significant level of 0.05 and 0.01 respectively by One-way ANOVA test. These experiments were performed by three independent biological replicates.

respectively ($p < 0.01$, Figure 3C). The average 1000-grain weight under N input of 0 kg ha^{-1} was 20.93 ± 2.30 g, which was slightly improved by 8.27% to 22.66 ± 2.48 g under 180 kg ha^{-1} ($p < 0.01$), but under 250 kg ha^{-1} N input 1000-grain weight decreased to 21.26 ± 2.76 g, however nonsignificant with that of moderate N input (180 kg ha^{-1}), indicating that high N input had limited impact on 1000-grain weight.

Impact of elevated N input on grain quality

Grain quality is one of the major objectives apart from yield in plant breeding, and in our study N input showed significant influence on rice plant growth and N content partitioning in different organs, grain quality under different N doses was studied as well. The overall quality index, ranged from 61.53% to 72.5%, did not change under low and moderate N doses, but significantly elevated at 250 kg ha^{-1} ($p < 0.01$, Figure 4A). Notably, the quality index of grain seed without N input (Control) was 72.06 ± 1.11%, which was significantly higher than low and moderate N input (120 and 180 kg ha^{-1} respectively), indicating that the N input reduced grain quality. As for starch, amylose was 14.19% ~ 17.88%, which is slightly higher than amylopectin under different N input, but both of them did not show significant difference among different N input ($p > 0.05$, Figures 4B, 4C). Fatty acid content of grain seed was 11.59% when no N was applied, and fatty acid content was significantly changed under 120 and 180 kg ha^{-1}, but elevated significantly by 14.8% under 250 kg ha^{-1} (Figure 4D), indicating the higher N input exceed 180 kg ha^{-1} is beneficial for fatty acid accumulation. Interestingly positive correlation was observed between fatty acid content and leaf chlorophyll content (Pearson correlation coefficient $r = 0.724$, $p < 0.01$, Table 2). In terms of seed protein content, it was found to be highly correlated to seed N content (Pearson correlation coefficient $r = 0.812$, $p < 0.01$, Table 2), and higher seed protein content in Q681 was observed when high N dose was applied, but not in Q1 and HHZ (Table 3).

Figure 3. Leaf and panicle characteristics in response to different N input.

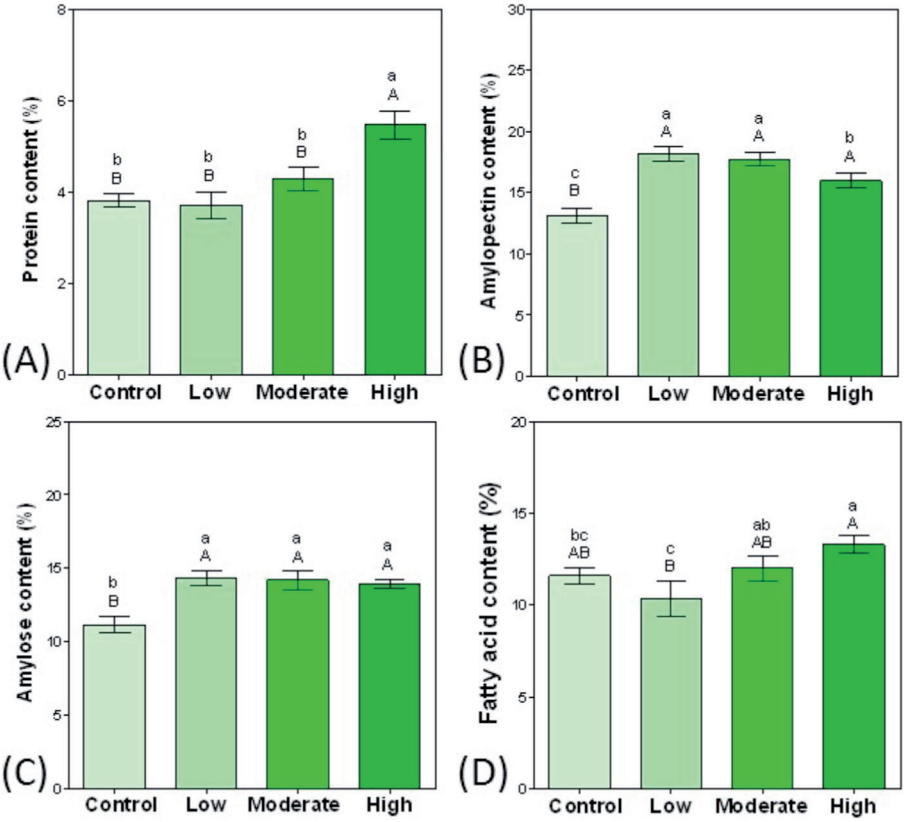


A-D: Leaf area (cm^2), leaf chlorophyll content (SPAD), panicle length (cm) and 1000-grain weight (g). Different N inputs are indicated by control (0 kg ha^{-1}), low (120 kg ha^{-1}), moderate (180 kg ha^{-1}), and high (250 kg ha^{-1}).
Lower-case and uppercase letters on the top of error bar (standard error) represent significant level of 0.05 and 0.01 respectively by One-way ANOVA test. These experiments were performed by three independent biological replicates.

GS activity variance to different N input

Urea is an organic fertilizer made of two amine group and a carbonyl group, which could be broken up and converted into ammonium in face of water (Kleiner, 1981). In higher plants, the ammonium ions (NH_4^+) are taken up by the roots and then assimilated into amide residue of glutamine (Gln) catalyzed by glutamine synthetase (GS). The Gln is then converted into glutamate (Glu) by glutamate synthase (GOGAT) (Lea and Miflin, 1974; Tabuchi et al., 2007; Gaur et al., 2012). As *OsGS1;1* and *OsNADH-GOGAT2* were mainly expressed in vascular tissues of mature leaf blades (Tabuchi et al., 2007; Tamura et al., 2011), the expression pattern of these two genes was investigated in our study. *OsGS1;1* in flag leaves expressed at very low level (0.33 ± 0.03, normalized by geometric average expression level of *actin*, *ubiquitin*, and *GAPDH*) in control flag leaves. *OsGS1;1*'s expression level was significantly increased by ~ 11.2 fold to 3.70 ± 0.47 under low N input at 120 kg ha^{-1} compared with control, and maintained unchanged when the N input at 180 kg ha^{-1} ($p > 0.05$, Figure 5A), but *OsGS1;1* decreased slightly at 250 kg ha^{-1} in all three genotypes tested ($p < 0.05$, Figure 5A, Table 3). This trend could be observed in the enzymatic activity of GS in Q681 and HHZ, while in Q1 the GS activity was higher in control but decreased when extra N was applied (Figure 5C), showed a genotype dependent manner. In flag leaves, the *OsNADH-GOGAT2* was expressed highly in control (0 kg ha^{-1}) but decreased dramatically under low and moderate N input, however high N input could induce the *OsNADH-GOGAT2* slightly (Figure 5B). With regard to each rice genotype, *OsGS1;1* and *OsNADH-GOGAT2* showed similar expression pattern (Figures 5D, 5E).

Figure 4. Grain quality indexes under different nitrogen input.



Grain quality index comparison under different nitrogen doses: protein content (A), amylopectin content (B), amylose content (C), fatty acid content (D) (Two-way ANOVA, n = 9).
Control: 0 kg N ha^{-1}; Low: 120 kg N ha^{-1}; Moderate: 180 kg N ha^{-1}; High: 250 kg N ha^{-1}.
Significance of 0.05 and 0.01 probability level is indicated by lower-case and uppercase letters on top of error bar (standard error). These experiments were performed by three independent biological replicates.

Response pattern to elevated N input among rice genotypes

Generally, HHZ has less 1000-grain weight than that of Q1 and Q618 under any N input conditions. When no extra N was applied (control), most of the phenotypic indexes related to plant growth, N partitioning and grain quality were similar among Q1, Q681, and HHZ, except that HHZ had less amylose content than Q681 (Table 3). And with the increase of N input, phenotypic indexes difference among rice genotypes expanded (5, 7, and 10 phenotypic indexes under 120, 180, and 250 kg ha^{-1}, respectively). Overall HHZ showed more tiller and panicle number, larger plant height, higher N content in root, leaf and culm, Q681 had better performance in tiller number and leaf N content, while Q1 were outstanding in panicle number, leaf area, and plant height (Table 3). These results indicated that although different rice genotype showed similar response pattern to N input, each genotype varied a lot in certain phenotypic indexes.

DISCUSSION

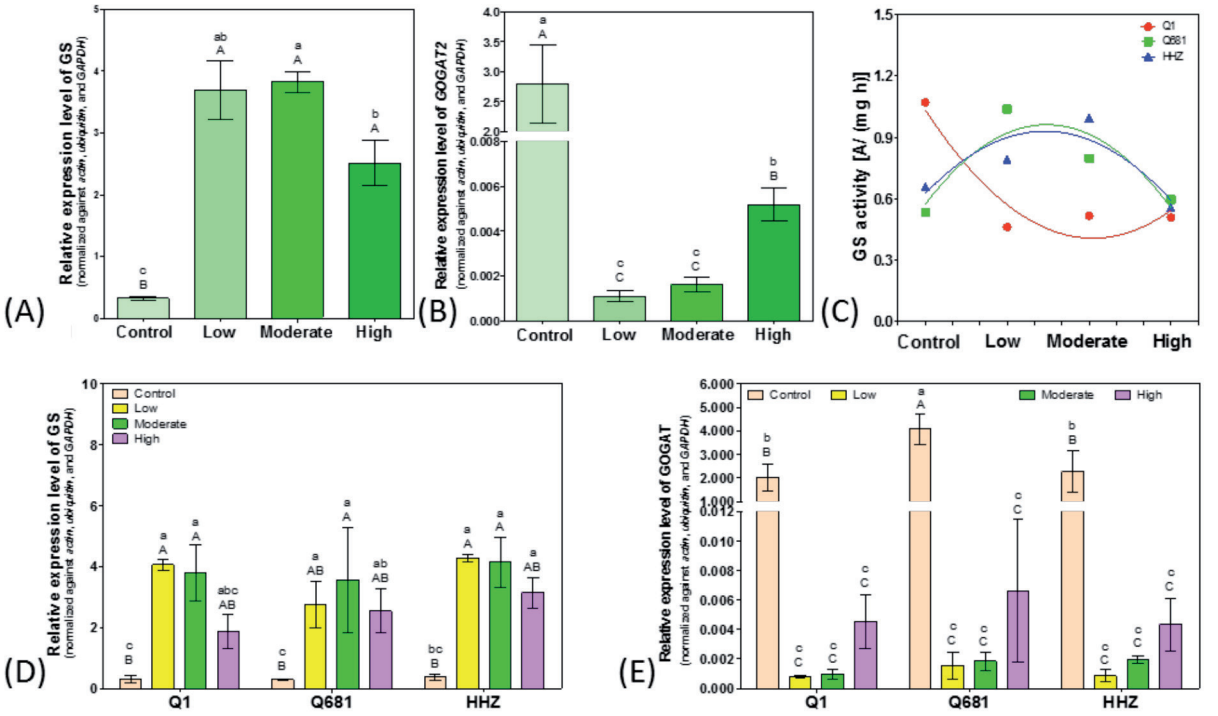
Nitrogen content in leaf, culm, seed and root were all significantly elevated under high N input (250 kg ha^{-1}), however the elevated N preferentially deposited in seed and leaf rather than culm and root (Figure 1), which is consistent with the report by Ye et al. (2014). The high N content in leaf could be explained by the increased *OsGS1;1*, *OsNADH-GOGAT2*, and GS activity in flag leaves, which probably contributed to the N accumulation in leaf (Li et al., 2016). Leaf is the organ where photosynthesis takes place, and the N content in leaf is closely related to photosynthetic process, and hence correlates to crop yield (Ata-Ul-Karim et al., 2017). The increase of N content in leaves and accompanied by

Table 3. Multiple comparison among different rice genotypes in terms of different phenotypic and molecular indexes.

Indexes	Bartlett test (test statistics; p value)	Levene test (test statistics; p value)	N supply	Control			Low			Moderate			High		
			Genotype	Q1	Q681	HHZ	Q1	Q681	HHZ	Q1	Q681	HHZ	Q1	Q681	HHZ
Leaf chlorophyll content	10.30; 0.504	0.41; 0.938	Mean±SE	29.80±0.87dC	31.99±0.52dC	30.94±0.19dC	32.30±1.27dC	37.72±1.60bcAB	32.39±0.74dC	35.66±1.31cBC	39.38±0.65abAB	36.12±1.36bcBC	35.46±0.93cdBC	42.12±1.63aA	39.13±0.42abAB
Tiller number	12.37; 0.336	0.44; 0.920	Mean±SE	7.56±1.87eD	7.89±0.62eD	7.89±0.22eD	18.11±2.16dC	19.00±1.84dC	21.22±0.78cdBC	24.56±1.85bcABC	22.22±2.11cdC	25.22±0.78bcABC	23.22±2.84cdBC	29.44±0.95abAB	30.67±1.58aA
Panicle number	16.72; 0.116	0.83; 0.615	Mean±SE	3.22±0.29dD	3.56±0.11dD	3.67±0.1dD	10.89±0.68cC	11.33±1.07cC	12.89±0.59cC	12.33±1.35cC	12.78±1.24cC	16.78±1.06bBC	16.56±0.87bC	17.33±0.88bAB	21.22±1.98aA
Panicle length, cm	13.52; 0.211	0.68; 0.742	Mean±SE	24.23±0.39bcBC	22.53±0.18cC	22.08±0.21cC	24.29±0.30bcBC	22.82±0.40cC	23.07±0.03cC	24.99±0.56bAB	24.17±0.13bcBC	23.22±0.24cC	26.17±0.47aA	24.98±0.62bAB	24.44±0.35bBC
Leaf area, cm^2	17.71; 0.089	0.71; 0.630	Mean±SE	23.27±1.63eEF	19.66±0.68eF	20.82±0.83eEF	46.09±3.76cdBCD	34.14±1.39dDEF	37.47±1.72dCDE	52.67±5.09bcBC	45.55±3.47cdBCD	39.28±2.37dCD	68.27±6.63aA	60.94±7.05abAB	43.83±2.25cdCD
Plant height, cm	8.10; 0.704	0.30; 0.979	Mean±SE	66.33±0.25fE	64.39±1.14fE	64.94±0.97fE	87.89±1.84deCD	84.72±1.08eD	86.78±1.24deD	97.89±2.53abAB	95.67±2.03abcAB	91.50±1.84cdBCD	99.94±2.17aA	94.33±1.86bcABC	96.89±1.25abAB
1000-grain weight, g	13.17; 0.282	0.50; 0.887	Mean±SE	23.30±0.30bA	23.16±0.84bA	16.34±0.26cB	24.89±0.28abA	23.08±0.83bA	17.38±0.29cB	25.54±0.54aA	24.72±0.15abA	17.71±0.25cB	24.40±0.59abA	23.62±0.72abA	15.75±1.18d
Leaves weight, g	15.38; 0.166	0.65; 0.773	Mean±SE	1.11±0.11eF	1.64±0.20eEF	1.57±0.38eEF	6.75±0.70cdCD	5.12±1.01dDE	7.77±1.12cdCD	8.87±0.80cBCD	9.23±1.35cBC	9.57±0.64bcBC	12.17±0.53abAB	13.62±1.78aA	14.03±0.99aA
Culm weight, g	24.00; 0.013	0.64; 0.777	Mean±SE	5.20±0.29fD	4.81±0.41fD	3.66±0.24fD	20.63±2.32eC	19.78±5.23eC	21.42±2.64deC	28.02±2.56bcdBC	23.55±0.80cdeC	29.49±1.22bcBC	34.55±1. 56bAB	43.66±1.65aA	41.47±3.12aA
N-content in root, %	13.52; 0.260	0.68; 0.742	Mean±SE	0.35±0.01cC	0.37±0.03cBC	0.47±0.04bcBC	0.44±0.01bcBC	0.40±0.05bcBC	0.51±0.01bB	0.46±0.04bcBC	0.38±0.02cBC	0.49±0.03bBC	0.47±0.02bcBC	0.51±0.05bB	0.73±0.05aA
N-content in leaf, %	25.57; 0.008	0.99; 0.481	Mean±SE	1.02±0.05abcAB	0.83±0.09bcAB	1.02±0.04abcAB	0.64±0.05cB	0.70±0.05cB	0.77±0.05cAB	0.67±0.05cB	0.73±0.10cAB	0.79±0.06cAB	0.92±0.12bcAB	1.31±0.27abAB	1.43±0.40aA
N-content in seed, %	37.49; 0.000	0.79; 0.648	Mean±SE	0.85±0.05bA	0.96±0.12abA	0.87±0.05abA	0.77±0.03bA	0.80±0.01bA	0.83±0.02bA	0.94±0.09abA	0.91±0.03abA	0.88±0.05abA	1.04±0.12abA	1.19±0.23abA	1.38±0.40aA
N-content in culm, %	37.36; 0.000	1.39; 0.240	Mean±SE	0.34±0.02bB	0.39±0.02bB	0.44±0.01bB	0.26±0.02bB	0.40±0.06bB	0.37±0.02bB	0.32±0. 02bB	0.35±0.02bB	0.34±0.03bB	0.40±0.02bB	0.48±0.01bB	0.75±0.22aA
Seed protein content, %	8.60; 0.659	0.43; 0.928	Mean±SE	3.77±0.34bcdBC	3.59±0.15cdBC	4.07±0.14bcdABC	3.16±0.21dC	3.29±0.34dBC	4.68±0.32abcABC	4.36±0.25bcdABC	3.66±0.38cdBC	4.82±0.46abcABC	4.95±0.82abAB	5.70±0.41aA	5.76±0.36aA
Amylopectin content, %	6.39; 0.846	0.40; 0.941	Mean±SE	13.04±1.27efB	12.73±0.52fB	13.60±1.34defB	16.89±0.48abcAB	19.15±1.43aA	18.42±0.64abA	18.28±0.56abA	16.19±0.39abcdAB	18.67±0.99abA	15.06±0.92cdefAB	15.86±1.12bcdeAB	17.02±1.09abcAB
Amylose content, %	15.88; 0.146	0.91; 0.547	Mean±SE	11.38±1.31deDE	12.14±0.35cdCDE	9.98±0.66eE	14.61±0.33abABC	12.58±0.20bcdBCDE	15.78±0.39aA	14.95±0.57aABC	12.42±1.31bcdBCDE	15.20±0.36aAB	14.40±0.54abABC	13.77±0.62abcABCD	13.56±0.11abcABCD
Fatty acid content, %	15.50; 0.169	0.33; 0.969	Mean±SE	11.57±0.92abAB	11.92±0.87abAB	11.27±0.79bcAB	8.73±1.07cdB	13.72±0.79abA	8.47±0.82dB	11.35±0.08bcAB	13.81±0.62abA	10.89±1.65bcdAB	12.27±1.17abAB	14.35±0.20aA	13.31±0.26abA
OsGS1 expression level	34.07; 0.000	0.78; 0.661	Mean±SE	0.30±0.11cB	0.29±0.02cB	0.38±0.10bcB	4.06±0.18aA	2.77±0.77aAB	4.28±0.13aA	3.79±0.92aA	3.55±1.73aA	4.14±0.82aA	1.86±0.56abcAB	2.55±0.73abAB	3.14±0.49aAB
OsGOGAT2 expression level	186.45; 0.000	1.68; 0.138	Mean±SE	2.0306±0.5716bB	4.0835±0.6578aA	2.2744±0.8911bB	0.0008±0.0001cC	0.0016±0.0009cC	0.0009±0.0004cC	0.0010±0.0003cC	0.0018±0.0006cC	0.0020±0.0002cC	0.0045±0.0018cC	0.0066±0.0048cC	0.0043±0.0018cC

SE: Standard error; Comparison with different among rice genotypes were highlighted in red. Significance of 0.05 and 0.01 probability level is indicated by lower-case and uppercase, respectively.

Figure 5. *OsGS1;1* and *OsNADH-GOGAT2* expression level and glutamine synthetase (GS) activity in flag leaves under different N inputs.



Relative expression of *OsGS1;1* (A) and *OsNADH-GOGAT2* (B) were normalized against *actin*, *ubiquitin*, and *GAPDH*. (C) Average GS activity [A/(mg × h)] among three rice genotypes under different N input. Best fit nonlinear regression curve was calculated by third order polynomial (cubic), R^2 was shown by the side of each curve. Relative expression of *OsGS1;1* (D) and *OsNADH-GOGAT2* (E) in each rice genotype under different N input. Control: 0 kg ha^{-1}; Low: 120 kg ha^{-1}; Moderate: 180 kg ha^{-1}; High: 250 kg ha^{-1}. Q1: 'Quanliangyou 1', Q681: 'Quanliangyou 681', HHZ: 'Huanghuazhan'. Significance of 0.05 and 0.01 probability level is indicated by lower-case and uppercase letters on top of error bar (standard error). These experiments were performed by three independent biological replicates.

the expansion of leaf area under high N input probably promoted the CO_2 assimilation and finally for plant growth and reproductive growth. This was further evidenced by the high positive correlation among N content in leaf, culm and seed, and also the increase of tiller number, plant height, panicle number, leaf weight, and culm weight (Table 3). Although the elevation of plant height and tiller number by high N input in rice has been documented in different studies (Gaur et al., 2012; Singh et al., 2014), in our study the plant height maximized when the N input was 180 kg ha^{-1}, and further increase of N input inhibited the plant to grow vertically, whilst the tiller number and panicle number continued increasing under N input of 250 kg ha^{-1}. Probably inner regulation process might exist in rice to prevent the plant height from growing taller and consequent lodging.

With respect to grain quality, high N input caused decrease in amylopectin content, but increase in the seed protein content, while amylose and fatty acid content remained unaffected (Figure 4). Previously it has been documented that amylose content of super rice cultivar could be decreased by applying N input (Wei et al., 2014). This is probably because that the N input adopted in Wei's research was 300 kg ha^{-1}, which was higher than 250 kg N ha^{-1} in our study. These results implied that grain quality is a kind of seed trait that could be improved by breeding with high N input.

The assimilation of ammonium ions in plant mainly depends on the cooperation between GS and GOGAT, which served as the key catalytic step during glutamate synthesis (Lea and Miflin, 1974). The GS expression under high N input has been somewhat inhibited but GOGAT was slightly induced, this was possibly because that high N could induce the conversion of glutamine to glutamate. This was consistent with the observation of the elevated raw seed protein content under high N input (Figure 4A).

We utilized three distinct rice genotypes, 'Quanliangyou 1', 'Quanliangyou 681' and 'Huanghuazhan', for response pattern analysis under high N input. Although different rice genotypes response similarly with the increase of N input, there was a big difference among rice genotypes (Table 3), suggesting that different N management practice was necessarily needed for these cultivars for better production. Unfortunately, no interaction was observed between rice genotype and different N input treatments (data not shown), which contradicted with the results by Singh et al. (2014). This is probably the result of the higher N input adopted (250 kg ha^{-1}) in our study. Taken together, these results implied that the response pattern of N partitioning, plant growth, *OsGS1;1* and *OsNADH-GOGAT2* expression to high N input was independent of the genotype.

CONCLUSIONS

Overall, the N content in leaf, culm, seed and root had all been increased but preferentially deposited in leaf and culm, which led to the increase in leaf area, leaf chlorophyll content, leaf weight and culm weight under high N input. The plant growth has also been significantly promoted in terms of tiller number, panicle number and length, but not the plant height and 1000-grain weight under high N input. In contrast, high N input did have negative impact on amylopectin content, while fatty acid and amylose content remained stable, but seed protein increased. Although three rice genotypes were investigated, the response pattern of all these phenotypic characteristics to high N input was independent of rice genotype. Taken together, the overview of N partitioning and the impact on plant growth and key genes expression involved in glutamate biosynthesis provided physiological and molecular foundation for rice breeding and cultivation.

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REFERENCES

Ali, M.B., Tripathi, R.D., Rai, U.N., Pal, A., and Singh, S.P. 1999. Physico-chemical characteristics and pollution level of lake Nainital (U.P., India): Role of macrophytes and phytoplankton in biomonitoring and phytoremediation of toxic metal ions. Chemosphere 39:2171-2182.

Ata-Ul-Karim S.T., Zhu Y., Liu X., and Cao Q., Tian Y., and Cao W. 2017. Comparison of different critical nitrogen dilution curves for nitrogen diagnosis in rice. Scientific Reports 7:42679.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72:248-254.

Cassman, K.G., Dobermann, A., and Walters, D.T. 2002. Agroecosystems, nitrogen-use efficiency, and nitrogen management. Ambio 31:132-140.

Dobermann, A., and Cassman, K.G. 2004. Environmental dimensions of fertilizer N: what can be done to increase nitrogen use efficiency and ensure global food security? p. 261-278. In Mosier, A.R., Syers, J.K., and Freney, J.R. (eds.) Agriculture and the nitrogen cycle: assessing the impacts of fertilizer use on food production and the environment. Island Press, Washington D.C., USA.

Fageria, N.K., dos Santos, A.B., and Coelho, A.M. 2011. Growth, yield and yield components of lowland rice as influenced by ammonium sulfate and urea fertilization. Journal of Plant Nutrition 34:371-386.

Gaur, V.S., Singh, U.S., Gupta, A.K., and Kumar, A. 2012. Influence of different nitrogen inputs on the members of ammonium transporter and glutamine synthetase genes in two rice genotypes having differential responsiveness to nitrogen. Molecular Biology Reports 39:8035-8044.

Giles, J. 2005. Nitrogen study fertilizes fears of pollution. Nature 433:791-791.

Hakeem, K.R., Ahmad, A., Iqbal, M., Gucel, S., and Ozturk, M. 2011. Nitrogen-efficient rice cultivars can reduce nitrate pollution. Environmental Science and Pollution Research 18:1184-1193.

Horneck, D.A., and Miller, R.O. 1998. Determination of total nitrogen in plant tissue. p. 75-83. In Kalra, Y.P. (ed.) Handbook of reference methods for plant analysis. CRC, Boca Raton, Florida, USA.

Kleiner, D. 1981. The transport of NH_3 and HN_4^+ across biological membranes. Biochimica et Biophysica Acta (BBA)-Reviews on Bioenergetics 639:41-52.

Ladha, J.K., Tirol-Padre, A., Reddy, C.K., Cassman, K.G., Verma, S., Powlson, D.S., et al. 2016. Global nitrogen budgets in cereals: A 50-year assessment for maize, rice, and wheat production systems. Scientific Report 6:19355.

Lea, P.J., and Miflin, B.J. 1974. Alternative route for nitrogen assimilation in higher plants. Nature 251:614-616.

Lee S., Jeong H., Lee S., Lee J., Kim S.J., Park J.W., et al. 2017. Molecular bases for differential aging programs between flag and second leaves during grain-filling in rice. Scientific Reports 7:8792.

Li, Y., Wang, M., Zhang, F., Xu, Y., Chen, X., Qin, X., and Wen, X. 2016. Effect of post-silking drought on nitrogen partitioning and gene expression patterns of glutamine synthetase and asparagine synthetase in two maize (*Zea mays* L.) varieties. Plant Physiology and Biochemistry 102:62-69.

Ling, Q., Huang, W., and Jarvis, P. 2011. Use of a SPAD-502 meter to measure leaf chlorophyll concentration in *Arabidopsis thaliana*. Photosynthesis Research 107:209-214.

Livak, K.J., and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25:402-408.

Mae, T. 1997. Physiological nitrogen efficiency in rice: Nitrogen utilization, photosynthesis, and yield potential. Plant and Soil 196:201-210.

Moraes, G.P., Benitez, L.C., do Amaral, M.N., Vighi, I.L., Auler, P.A., da Maia, L.C., et al. 2015. Evaluation of reference genes for RT-qPCR studies in the leaves of rice seedlings under salt stress. Genetics and Molecular Research 14:2384-2398.

Nestler A., Berglund M., Accoe F., Duta S., Xue D., Boeckx P., et al. 2011. Isotopes for improved management of nitrate pollution in aqueous resources: review of surface water field studies. Environmental Science and Pollution Research 18:519-533.

Rahn, C.R., Bending, G.D., Lillywhite, R.D., and Turner, M.K. 2009. Co-incorporation of biodegradable wastes with crop residues to reduce nitrate pollution of groundwater and decrease waste disposal to landfill. Soil Use and Management 25:113-123.

Samonte, S.O.P., Wilson, L.T., Medley, J.C., Pinson, S.R.M., McClung, A.M., and Lales, J.S. 2006. Nitrogen utilization efficiency. Agronomy Journal 98:168-176.

Shrawat, A.K., Carroll, R.T., DePauw, M., Taylor, G.J., and Good A.G. 2008. Genetic engineering of improved nitrogen use efficiency in rice by the tissue-specific expression of alanine aminotransferase. Plant Biotechnology Journal 6:722-732.

Singh, H., and Verma, A. 2013. Characterization and screening of high nitrogen efficient rice genotype to curtail environmental pollution. International Journal of Agriculture and Environment and Biotechnology 6:23-30.

Singh, H., Verma, A., Ansari, M.W., and Shukla, A. 2014. Physiological response of rice (*Oryza sativa* L.) genotypes to elevated nitrogen applied under field conditions. Plant Signaling and Behavior 9:e29015.

Simplicio, N.C.S., Muniz, D.H.F., Rocha, F.R.M., Martins, D.C., Dias, Z.M.B., Farias, B., et al. 2016. Comparative analysis between ecotoxicity of nitrogen-, phosphorus-, and potassium-based fertilizers and their active ingredients. Toxics 5:2.

Smil, V. 2001. Nitrogen in crop production. An account of global flows. Global Biogeochemical Cycles 13:647-662.

Tabuchi, M., Abiko, T., and Yamaya, T. 2007. Assimilation of ammonium ions and reutilization of nitrogen in rice (*Oryza sativa* L.) Journal of Experimental Botany 58:2319-2327.

Tabuchi, M., Sugiyama, K., Ishiyama, K., Inoue, E., Sato, T., Takahashi, H., et al. 2005. Severe reduction in growth rate and grain filling of rice mutants lacking *OsGS1;1*, a cytosolic glutamine synthetase1;1. The Plant Journal 42:641-651.

Tamura, W., Kojima, S., Toyokawa, A., Watanabe, H., Tabuchi-Kobayashi, M., Hayakawa, T., et al. 2011. Disruption of a novel NADH-glutamate synthase2 gene caused marked reduction in spikelet number of rice. Frontiers in Plant Science 2:57.

Wang, P., Zhou, G., Cui, K., Li, Z., and Yu, S. 2012. Clustered QTL for source leaf size and yield traits in rice (*Oryza sativa* L.) Molecular Breeding 29:99-113.

Wei, H.Y., Wang, Y.J., Meng, T.Y., Ge, M.J., Zhang, H.C., Dai, Q.G., et al. 2014. Response of yield, quality and nitrogen use efficiency to nitrogen fertilizer from mechanical transplanting super japonica rice. Ying Yong Sheng Tai Xue Bao 25:488-496.

Ye, Y., Liang, X., Chen, Y., Li, L., Ji, Y., and Zhu, C. 2014. Carbon, nitrogen and phosphorus accumulation and partitioning, and C:N:P stoichiometry in late-season rice under different water and nitrogen managements. PLOS ONE 9:e101776.