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Gene Expression, Enzyme Activity, Nitrogen Use Efficiency, and Yield of Rice Affected by Controlled-Release Nitrogen

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Cite This: *ACS Omega* 2023, 8, 23772−23781

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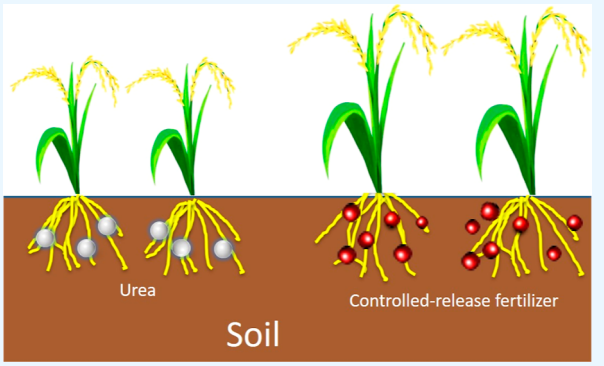
ABSTRACT: Controlled- or slow-release urea can improve crop nitrogen use efficiencies and yields in many agricultural production systems. The effect of controlled-release urea on the relationships between levels of gene expression and yields has not been adequately researched. We conducted a 2 year field study with direct-seeded rice, which included treatments of controlled-release urea at four rates (120, 180, 240, and 360 kg N ha^{-1}), a standard urea treatment (360 kg N ha^{-1}), and a control treatment without applied nitrogen. Controlled-release urea improved the inorganic nitrogen concentrations of root-zone soil and water, functional enzyme activities, protein contents, grain yields, and nitrogen use efficiencies. Controlled-release urea also improved the gene expressions of nitrate reductase [NAD(P)H] (EC 1.7.1.2), glutamine synthetase (EC 6.3.1.2), and glutamate synthase (EC 1.4.1.14). With the exception of glutamate synthase activity, there were significant correlations among these indices. The results showed that controlled-release urea improved the content of inorganic nitrogen within the rice root zone. Compared with urea, the average enzyme activity of controlled-release urea increased by 50−200%, and the relative gene expression was increased by 3−4 times on average. The added soil nitrogen increased the level of gene expression, allowing enhanced synthesis of enzymes and proteins for nitrogen absorption and use. Hence, controlled-release urea improved the nitrogen use efficiency and the grain yield of rice. Controlled-release urea is an ideal nitrogen fertilizer showing great potential for improving rice production.



INTRODUCTION

Nitrogen is the most important nutrition for plant growth, productivity, and metabolism, and it signals plant gene expression.[1−3] Nitrate (NO_3^-) and ammonium (NH_4^+) are two main inorganic forms of nitrogen, which are directly absorbed by the root system and affect the plant enzymes.[4,5] Kiran et al. and Atere et al. found that crop yields are strongly dependent on the levels of supplied nitrogen fertilizers;[6,7] however, less than 40% of the applied nitrogen was taken up by the crops.[8,9] Increasing nitrogen fertilization levels above the requirements for maximum plant yields results in increased expenditures without increased profits, or "diminishing returns"; this practice also reduces nitrogen use efficiencies.[10−12] Reducing nitrogen losses and improving nitrogen use efficiencies are essential for sustainable agricultural development.[13]

Controlled-release urea has been applied to a variety of crops, resulting in numerous advantages.[14,15] They are coated with a polymer to control or lessen the release rate of nitrogen, which reduces nutrient losses and groundwater pollution while increasing nitrogen use efficiencies and grain yields at the plant's physiological level.[16,17] Many studies have proved that it is because the nitrogen release patterns and rates from controlled-release urea match well with the crop plants' requirements.[18−20] These nitrogen release patterns affect soil inorganic nitrogen concentrations which are the signal molecules in plants. In the laboratory, many have studied the effects of different nitrogen concentrations on the levels of gene expression in Arabidopsis.[8,21] Because of the increasing applications of nitrogen fertilizers, investigating these effects on grain crops such as rice, corn, and maize in the greenhouse or laboratory has become a topic of considerable research effort.[22−25] However, under field conditions, these topics have been investigated much less frequently. It is still unknown how the release patterns of controlled-release urea may change the levels of gene expression and enzyme synthesis of rice. The molecular mechanisms behind how controlled-release urea improves plant nitrogen use efficiencies would seem well worth researching.

Received: March 29, 2023
Accepted: June 1, 2023
Published: June 20, 2023

In the present study, direct-seeded rice was used to determine the molecular mechanisms of controlled-release urea for improving plant nitrogen use efficiencies and grain yields. Rice (*Oryza sativa L.*) is a primary food source and staple crop for over 50% of the world's population and about 65% of the population of China.[26,27] Planting rice crops is usually performed mechanically either by transplanting plantlets or directly sowing seeds. Hence, the direct seeding of rice offers many advantages because of the simpler propagation and reduced production costs.[28,29] Objectives of the present study were to investigate the effects of controlled-release urea on the nitrogen use efficiency and yield of directed-seeded rice and reveal the reason that controlled-release urea improved the nitrogen use efficiency and yield of directed-seeded rice by detecting the levels of enzyme activity, protein content, and gene expression related to nitrogen and determining how directed-seeded rice absorbs and uses nitrogen. Correlations between soil inorganic nitrogen, enzyme activity, protein content, gene expression, nitrogen use efficiency, and/or grain yields also were determined. The hypothesis was that controlled-release urea increases gene transcription, functional enzyme synthesis, and nitrogen use efficiencies of direct-seeded rice, thereby improving its production.

EXPERIMENTAL SECTION

Experimental Materials. Field experiments were conducted during 2016−2017 at Yaozhuang, Yutai City Basin, Shandong Province, China (Figure S1, 35°08′ N, 116°53′ E). This area has a temperate, subhumid to humid monsoon climate with a mean air temperature of 13.2 °C, an annual precipitation of 647−900 mm, and a rainy season lasting from June to October. The dominant soil type is hydromorphic paddy soil.[15] The main properties of the top 20 cm of soil at the test site included the following: pH, 8.2 (the ratio of soil to water was 1:2.5); soil total nitrogen concentration, 1.56 g kg^{-1}; available phosphorus concentration, 10.56 mg kg^{-1}; available potassium concentration, 156.32 mg kg^{-1}; organic matter concentration, 18.75 g kg^{-1}; clay, 18.52%; silt, 72.62%; and sand, 8.86%.

We used the rice cultivar, *O. sativa* 'Runnong11', which has resistance to pests and diseases and produces high grain yields. Seeds were planted in the field on June 15 of both 2016 and 2017, with the grain harvested on October 12, 2016, or October 22, 2017.

Experimental Design. The rice field tests included six total treatments each with three replications in a randomized block design. The control treatment received no nitrogen fertilizer, but there were five nitrogen-fertilized treatments: one of standard urea (U, 360 kg N/ha) and four of controlled-release urea (CRU1, 120 kg N ha^{-1}; CRU2, 180 kg N ha^{-1}; CRU3, 240 kg N ha^{-1}; and CRU4, 360 kg N ha^{-1}, respectively). The controlled-release urea in these treatments was coated with a thermoplastic resin, which had been previously recycled from plastics. The control treatment was provided with a mono-potassium phosphate mix (52% P_2O_5 and 34.0% K_2O) and potassium sulfate but no nitrogen. In addition to the nitrogen treatment applications, the U and controlled-release urea treatments were fertilized with sulfate and 50% K_2O to provide potassium and diammonium phosphate to provide both phosphorus and quick-acting nitrogen. The nitrogen applications for the controlled-release urea treatments (CRU1, CRU2, CRU3, and CRU4) were all applied when the seeds were sown. For the standard urea treatment (U); however, 25% was initially applied at planting, while three more applications of 25% each were made at 20, 30, and 50 d, after planting, respectively. There were 18 rectangular plots, each 6.0 × 3.3 m (20 m^2) and representing one treatment and one replication. Each plot was divided by a ridge (40 cm wide and 40 cm high). There were 14 rows (planting beds) in each plot whereby the rectangular widths were spaced 25 cm apart. Rice seeds were planted at 23.6 g per row or 330 g per plot.

SAMPLING AND ANALYTICAL TECHNIQUES

During both years, soil samples were collected from each plot at the milk stage from a depth of 0−20 cm depth using a drill (2.0 cm diameter × 100 cm length). We pulled out the plants in the three zones, then removed the excess soil, and collected the soil near the roots as soil samples from the rhizosphere. The samples were air-dried and then passed through sieves of 2.0 mm and 0.25 mm for further analyses. Soil NO_3^--N and NH_4^+-N were extracted from each sample using 0.01 mol/L of $CaCl_2$ followed by determination of concentrations in the extract solution using an AA3-A001-02E autoanalyzer (Bran-Luebbe Co., Norderstedt, Germany).[30] Inorganic soil nitrogen concentration within the root zone of rice plants was considered to be the sum of the concentrations of NO_3^--N and NH_4^+-N.

Two suction lysimeters were installed in each plot at a 20 cm soil depth to gather water samples, which were then transferred to 50 mL plastic centrifuge tubes using an injector, followed by placement of the tubes in a bubble chamber with ice bags for storage. Water samples were then brought to the laboratory, and the concentrations of NO_3^--N and NH_4^+-N were determined using similar methods with soil samples.[31] Similar to the analyses of soil samples, inorganic nitrogen concentrations of water within rice root zones were considered to be the sum of the NO_3^--N and NH_4^+-N concentrations.

Rice plant weights were measured from the seedling to mature stages, followed by determination of the growth rates (Table 1); the growth rate was calculated by the plant weight divided by the growth days. Five rice plant samples were collected randomly from each plot by cutting off the above-ground portions at the mature stage. Plants with grains were dried in a drying oven at 75 °C to a constant weight before being weighed and sieved (100-mesh). Nitrogen concentrations were measured using an automatic chemical analyzer Smartchem 200 (AMS Alliance, Guidonia, Italy).[19] At maturity, the yields of total biomass and grains were measured by hand from a 1 m^2 representative area of each plot. Based on sums of dry matter and nitrogen concentration for each plot, their above-ground nitrogen uptakes were determined. The total nitrogen use efficiency was calculated using the formula of Devkota et al.[32] (eq 1).

$$\begin{aligned}&\text{Nitrogen use efficiency}(\%)\\&= [(\text{N uptake from the nitrogen treatment})\\&\quad - (\text{N uptake from the control treatment})]\\&\quad / [\text{Applied fertilizer N in the nitrogen treatment}\\&\quad \times 100]\end{aligned} \tag{1}$$

Based on data for the growth rates of rice plants (Table 1), immature leaf tips were gathered at the milk stage, put into a

https://doi.org/10.1021/acsomega.3c02113
ACS Omega 2023, 8, 23772−23781

Table 1. Rates of Mass Accumulation in Rice Plants during 2016−2017 [kg/ha)/d]

		growth stage[b]				
year	treatment[a]	seedling 0−20 d	tillering 20−30 d	panicle initiation 30−50 d	milk 50−80 d	maturity 80−120 d
2016	control	1.4d	49e	31e	53c	13c
	U	3.0b	76c	82c	114b	47a
	CRU1	2.5c	67d	47b	114b	28b
	CRU2	2.9b	77c	74c	136b	57a
	CRU3	3.3a	91b	104b	120b	50a
	CRU4	3.3a	100a	127a	149a	47a
2017	control	1.4e	49d	40d	65e	16c
	U	2.4c	79c	74c	109b	44b
	CRU1	1.9d	64b	47d	77de	26c
	CRU2	2.3c	76c	72c	88cd	56a
	CRU3	3.0b	88b	101b	97bc	62a
	CRU4	3.2a	95a	129a	147a	21c

[a]Control: no nitrogen fertilizer; U: standard urea applied at 360 kg of N/ha; controlled-release urea was applied at 120 CRU1, 180 CRU2, 240 CRU3, or 360 CRU4 kg of N/ha. [b]Means within each column followed by different lowercase letters were significantly different based on a one-way ANOVA followed by Duncan's multiple-range tests $P < 0.05$).

kettle with liquid nitrogen, and then brought to the laboratory for qPCR tests and enzyme activity analyses. According to the methods reported by Miller et al., the levels of nitrogen metabolism activity were measured for the following enzymes: glutamine synthetase (GS), glutamate synthase, also known as glutamine-2-oxoglutarate amidotransferase (GOGAT), and nitrate reductase (NR).[8]

For isolating and purifying RNA samples, synthesizing cDNA, and using real-time PCR, the protocols followed were the same as those in the previous study.[21] Five kinds of genes related to nitrogen metabolism in rice were observed for analyses: *OsNIA* for NR, *OsGS*1 and 2 for GS, and *OsGLN1*.1 and 1.2 for GOGAT. *OsActin* served as the internal reference gene along with different primers (Table 2).

In addition, rice plant heights were found using a tape measure, and chlorophyll density (SPAD) values were measured using a handheld chlorophyll meter (SPAD-502; Minolta, Tokyo, Japan).

Table 2. Primers Used to Study Rice Genes Related to Nitrogen Metabolism

primer[a]	nucleic acid sequence 5′ to 3′)
*OsNIA*53130-F	AGATATACTTCAAGGGCGAGGA
*OsNIA*53130-R	CCCTTGATGTCGATGGTG
OsGS1-F	GGAATGTATGCGGTGATGGT
OsGS1-R	TGAAATCCAGCAGGGAACTG
OsGS2-F	GTGATCAAGAAGGCAATCCTAAAC
OsGS2-R	CTCGTGTAAACCTGTCAACCT
OsGLN1.1-F	GAACGGCAAGGGCTACTT
OsGLN1.1-R	CTTCCAGATGATGGTGGTCTC
OsGLN1.2-F	CCGACATCAACACCTTCAAATG
OsGLN1.2-R	GCCTCCTGTCCTCGAAGTA
OsActin-F	GGAACTGGTATGGTCAAGGC
OsActin-R	AGTCTCATGGATAACCGCAG

[a]F represents the front end, and R represents the rear end of each primer.

STATISTICAL ANALYSES

For data processing and drawing figures, Microsoft Excel 2010 and SigmaPlot 12.5 were used, respectively. Analyses of variance followed by Duncan mean separation tests were performed using Statistical Analysis Systems version 9.2 (SAS 2012). Redundancy analyses (RDAs) combined with CANOCO 5.0 software (Microcomputer Power, Ithaca, NY, USA) provided an ordination method and multivariate statistical approach used in the present study. RDAs helped to analyze data for enzyme activity, gene expression, and the effects of multiple environmental effects, such as inorganic nitrogen concentrations in soil and water.

RESULTS AND DISCUSSION

Soil and Water Concentrations of Inorganic Nitrogen within Rice Root Zones. Effects among treatments appeared similar during 2016 and 2017 for the levels of inorganic nitrogen within the soil and water of rice root zones (Figure 1). For water samples in 2016, the control treatment resulted in significantly higher levels of inorganic nitrogen than CRU1 and statistically the same levels as U. Otherwise, the levels of inorganic nitrogen from soil and water were all significantly lower in the control than all other treatments during both years. In 2016, the soil inorganic nitrogen concentration of CRU1 was statistically the same as that for U. However, during both years, soil and water nitrogen concentrations were otherwise significantly higher for U (360 kg of N ha^{-1}) than for CRU1 (120 kg of N ha^{-1}), the lowest concentration of controlled-release urea. On the other hand, the most concentrated controlled-release urea, CRU4 (360 kg of N ha^{-1}), yielded significantly higher nitrogen levels than U, CRU1, or the control during both years and for both water and soil samples. CRU4 nitrogen levels were significantly higher than those from all other treatments in soil and water samples from both years except for 2016 water samples when they were statistically the same as the two immediately lower concentrations: CRU3 (240 kg of N ha^{-1}) and CRU2 (180 kg of N ha^{-1}). Both these intermediate treatments, along with CRU4, led to significantly higher nitrogen levels than U, CRU1, or the control in both water and soil samples during both years. The second-highest concentration (CRU3) was significantly higher than the third-highest (CRU2) in soil nitrogen in 2017, unlike in 2016, when the two treatments yielded the same levels in both kinds of samples.

Activities of the Three Enzymes. In both 2016 and 2017, the NR activity was significantly higher in CRU3 and CRU4 than in U, which was significantly higher than that in CRU2, which was significantly higher than that in CRU1 while that of the control was significantly lower than in all other treatments (Figure 2a). During both years, the activity of GS from the CRU4 treatment was significantly higher than that in all other treatments while those of CRU3, CRU2, and U were more intermediate with each significantly higher than that of CRU1 or the control (Figure 2b). The nitrogen application rates of the five treatments were 35.74, 67.21, 60.85, 55.90, and 53.73% (U, CRU1, CRU2, CRU3, and CRU4, respectively) in 2017, which were similar to the rates in 2016.[15] Although the nitrogen application rate of the U treatment was just half the rate provided in CRU2, the two treatments had almost the same effect on the activities of GS. During both years, the control treatment, without added nitrogen, resulted in significantly lower GOGAT activities than all the other

https://doi.org/10.1021/acsomega.3c02113
ACS Omega 2023, 8, 23772−23781

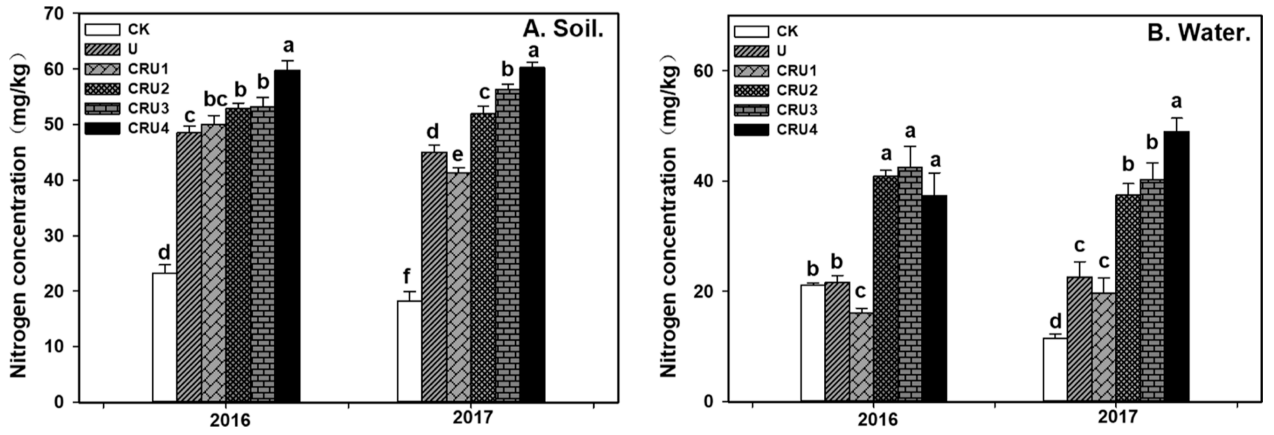


Figure 1. Inorganic nitrogen concentrations from samples of soil and water taken within rice root zones 0−20 cm deep during the milk stages of 2016 and 2017. Treatments included no applied nitrogen control, CK; standard urea at 360 kg of N/ha U; and treatments that were fertilized with controlled-release urea at 120, 180, 240, and 360 kg of N/ha CRU1, CRU2, CRU3, and CRU4, respectively.

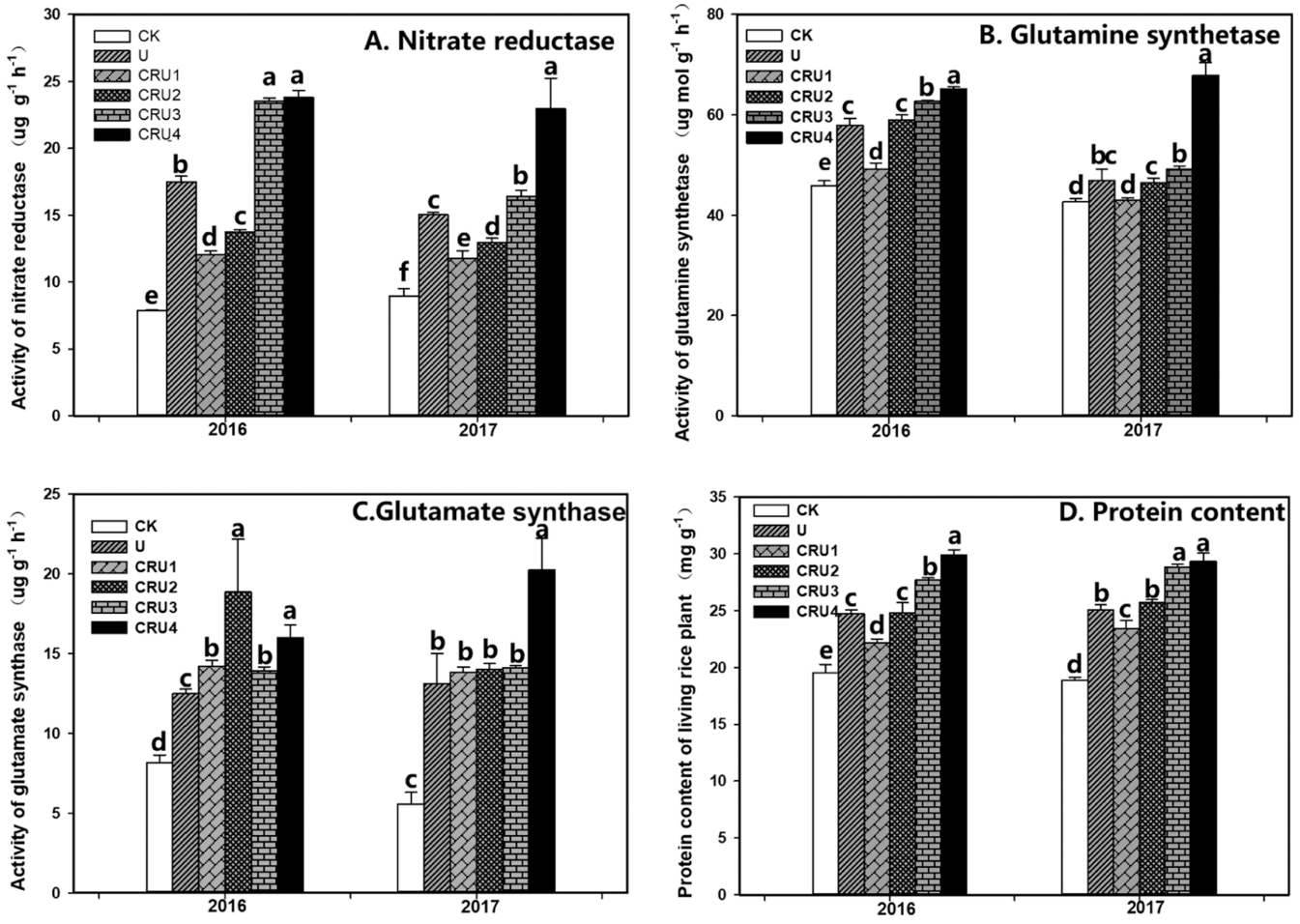


Figure 2. Enzyme activities and protein content within rice leaves during the milk stages of 2016 and 2017. Treatments included no applied nitrogen control, CK; standard urea at 360 kg of N/ha U; and treatments that were fertilized with controlled-release urea at 120, 180, 240, and 360 kg of N/ha CRU1, CRU2, CRU3, and CRU4, respectively.

treatments, whereas CRU4 was consistently in the highest group statistically (Figure 2c). Except for CRU2, which was also in the highest group statistically in 2016, the remaining treatments were statistically intermediate between the control and CRU4 during both years for GOGAT activity. When compared with analyses of the foregoing enzymes, rice protein contents tended to produce similar results, which were generally more consistent between 2016 and 2017 (Figure 2d). During both years, CRU4 and CRU3 each were in the highest group statistically and significantly higher than CRU2 or U, which were significantly higher than CRU1, with the control significantly lower than all the other treatments. Redundancy analyses were used to investigate relationships between enzyme activities and inorganic nitrogen concen-

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ACS Omega 2023, 8, 23772−23781

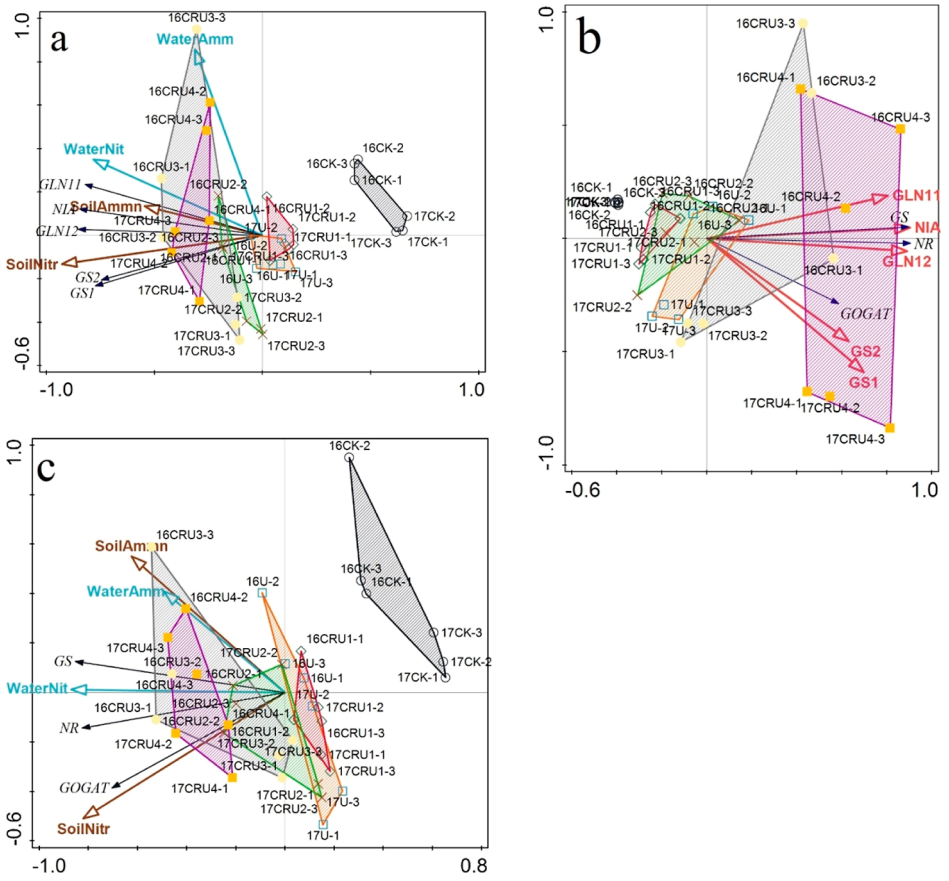

Figure 3. Redundancy analysis correlation triplots showing the relationships between (a) inorganic nitrogen concentration and amount of gene expression, (b) enzyme activity and the amount of gene expression, and (c) inorganic nitrogen concentration and enzyme activity. Solid lines with solid arrowheads show changes in dependent variables, while solid lines with empty arrowheads denote changing environmental factors. (a) *NIA*, *GS1* and *2*, and *GLN1.1* and *1.2* mean the gene expression of nitrate reductase, glutamine synthetase, and glutamate synthase; (b) NR, GS, and GOGAT mean the enzyme activity of nitrate reductase, glutamine synthetase, and glutamate synthase; (c) SoilAmmn, Soil Nitr, WaterAmm, and WaterNit mean the concentrations of NO_3^--N and NH_4^+-N in soil and water. Circles, squares, diamonds, spots, and crosses indicate different treatments during the two seasons. Each shaded figure indicates one treatment, and a circle and point label shows each replication. Each treatment label begins with "16" for 2016 or "17" for 2017; these are followed by codes for different treatments including no applied nitrogen control, CK; standard urea at 360 kg of N/ha U; and treatments of controlled-release urea at 120, 180, 240, and 360 kg of N/ha CRU1, CRU2, CRU3, and CRU4, respectively.

trations. Results of redundancy analyses suggested that the first two axes provided a good representation of all the enzyme activity variables in the different treatments (Figure 3; Table 3). Based on Monte Carlo permutation tests, the concentrations of soil ammonia and nitrate in water and soil each had significant effects on enzyme activities. The explanations of the three environmental factors were 51.8, 11.9 and 6.4% ($p \leq 0.01$). Except for GOGAT activity, there were significant correlations among soil inorganic nitrogen concentrations, enzyme activities, and grain yields (Table 4).

Table 3. Additional Data Related to Results from Redundancy Analyses Shown in Figure 3

figure	sum %[a]	axis	λ as %[b]	λ as cumulative %	λ as cumulative % of the sum of all canonical eigenvalues
3a	69.0	1	64.19	64.19	93.02
		2	3.87	68.06	98.63
3b	76.3	1	74.55	74.55	97.71
		2	1.38	75.93	99.52
3c	72.9	1	68.26	68.26	93.69
		2	3.61	71.87	98.65

[a]Of the total of all canonical eigenvalues. [b]λ is the standard deviation of the scores.

Effects of Nitrogen on Gene Expression. Five genes related to enzyme synthesis were measured in this study. The results of *OsNIA* gene products, which help to synthesize NR, were similar during 2016 and 2017 (Figure 4a). During both years, *OsNIA* gene activity from the CRU4 treatment was significantly higher than from all the other treatments, while CRU3, CRU2, and U were each more intermediate and significantly higher than CRU1 or the control (Figure 4a). The expression of CRU2 and U was statistically the same in both years. Both *OsGS1* and *OsGS2* could guide the synthesis of GS, The expression of *OsGS1* genes which produce GS was similar to those coding for *OsNIA* during 2016−2017 because *OsGS1* gene product activity from the CRU4 treatment was significantly higher than from all the other treatments (Figure 4b). During both years for *OsGS1*, CRU3 resulted in significantly higher activity levels than all remaining treatments including CRU2 and U, which were each significantly higher than that of the control. Similarly, for both test years with GS 2

https://doi.org/10.1021/acsomega.3c02113
ACS Omega 2023, 8, 23772−23781

Table 4. Results of Correlation Analyses Showing Correlation Coefficients and Their Levels of Significance for Traits That Were Measured or Calculated from the Controlled-Release Urea Treatments at the Milk Stage during 2016 and 2017[a]

trait[b]	N	Yield	NUE	NR	GS	GOGAT	Protein	*NIA*	*GS*1	*GS*2	*GLN*1.1	*GLN*1.2
N	1											
yield	0.726**	1										
NUE	−0.664**	−0.632**	1									
NR	0.702**	0.703**	−0.730**	1								
GS	0.728**	0.722**	−0.538**	0.860**	1							
GOGAT	0.411*	0.3061^{NS}	-0.2253^{NS}	0.317^{NS}	0.566**	1						
protein	0.762**	0.730**	−0.875**	0.768**	0.626**	0.2219^{NS}	1					
OsNIA	0.710**	0.713**	−0.536**	0.775**	0.787**	0.3258^{NS}	0.760**	1				
OsGS1	0.686**	0.534**	−0.811**	0.637**	0.552**	0.414*	0.788**	0.569**	1			
*OsGS*2	0.767**	0.592**	−0.803**	0.571**	0.456*	0.3008^{NS}	0.825**	0.593**	0.935**	1		
OsGLN1.1	0.659**	0.662**	−0.508*	0.795**	0.741**	0.2504^{NS}	0.712**	0.927**	0.530**	0.569**	1	
OsGLN1.2	0.803**	0.764**	−0.800**	0.888**	0.783**	0.3143^{NS}	0.882**	0.798**	0.808**	0.792**	0.843**	1

[a]Significant at $P < 0.05$ * or $P < 0.01$ **. The source data resulted from pooling all controlled-release urea treatments from both years; n = 24. [b]Soil inorganic nitrogen, N; grain yield, Yield; nitrogen use efficiency, NUE; enzyme activities including nitrate reductase NR, glutamine synthetase GS, and glutamate synthase GOGAT; protein content of rice leaves, Protein, and the expression levels for genes controlling nitrogen metabolism in rice *NIA*, *GS*1, *GS*2, *GLN*1.1, and *GLN*1.2.

(*OsGS2*) and with *OsGLN1.1*, CRU4 showed significantly higher gene-product expression levels than CRU3, which was significantly higher than that of CRU2 or U, which were each significantly higher than that of CRU1 or the control (Figure 4c,d). Similarly, for both years of tests on *OsGLN1.2*, CRU4 showed significantly higher amounts of gene expression than CRU3, which was significantly higher than that of CRU2, CRU1, or U, which were each significantly higher than that of the control (Figure 4e). Generally, *OsGLN1.1* and *OsGLN1.2* can trigger the synthesis of GOGAT. There were no significant differences between CRU2 and U during both years for *OsNIA* or in 2016 for *OsGS1, OsGS2,* or *OsGLN1.2*. CRU1 was significantly lower than U during both years for all five gene products except for *OsGLN1.2* in 2017. A redundancy analysis biplot indicated there were significant correlations between the levels of soil nitrate or ammonia and the levels of expression for all five gene products (Figure 3a). Cosines of the angles between the arrows showing levels of enzyme activity and those showing gene expression permitted their linear correlations to be plotted. Specifically, linear correlations were determined between *OsNIA* and NR, *OsGLN1.1* or *OsGLN1.2* each versus GOGAT, and *OsGS1* or *OsGS2* each versus GS (Figure 3b).

Treatment Effects on Soil Inorganic Nitrogen Concentrations. Within the rice-plant root zones (0−20 cm), soil and water inorganic nitrogen concentrations were affected by the kinds and amounts of nitrogen fertilization with controlled-release urea leading to the highest nitrogen contents. Rice production involves a large nitrogen requirement for plant growth and high nitrogen losses from leaching. Because standard urea (U, 360 kg of N/ha) did not provide a continuous supply of nitrogen, its soil levels were lower than those from treatments supplied with the three highest concentrations of controlled-release urea (CRU2, CRU3, and CRU4) in the rice milk stage. Hence, controlled-release urea treatments improved soil nitrogen contents presumably because the release of nitrogen was made continuous by a coating on the fertilizer grains. Zhang et al. (2016) reported similar results, noting that when compared to standard urea,[15] controlled-release urea improved rice nitrogen use efficiencies and grain yields.

Fertilizer Treatment Effects on Enzyme Activities. NR, GS, and GOGAT are key enzymes involved in plant nitrogen metabolism 3031. Nitrate is absorbed by the roots but is mainly transferred to the shoots.[33,34] In the shoots, nitrate is reduced to nitrite by NR followed by further reduction to ammonium. For ammonium, most of it is taken up by the roots; then, GS/GOGAT changes it to amino acids.[8] The leaves of rice and other plant species serve as an interim transfer station for nitrogen. Here, with the help of enzymes such as NR and GS/GOGAT, nitrogen is converted to a form usable by developing seeds.[1] We found that compared with standard urea, controlled-release urea improved the activities of enzymes including NR, GS, and GOGAT in the rice leaves. This may have resulted from increases in the amount of inorganic soil nitrogen within rice root zones to levels greater than that from standard urea in the milk stage. Strong correlations were found between concentrations of inorganic soil nitrogen and the activities of these enzymes, thereby supporting this conclusion (Table 4). Yang et al. similarly, reported that with increasing concentrations of inorganic soil nitrogen,[18] the activities of NR, GS, and GOGAT also increased. In our study, the controlled-release urea treatments improved the protein contents and activities of GS, GOGAT, and NR. Hence, the controlled-release urea treatments can improve rates of nitrogen uptake and use compared with standard urea treatments (U, 360 kg of N ha^{-1}).

Effects of Controlled-Release Urea on the Levels of Gene Expression. The levels of gene expression often have been found to be affected by external stimuli.[35] In the present study, the highest two concentrations of controlled-release urea often resulted in greater levels of gene expression than from the standard urea, which caused crops to generally use the extra nitrogen. Amounts of gene expression for products involving nitrogen metabolism are often affected by concentrations of root-zone soil nitrogen, which therefore provides a signal to plants.[1,2] The highest two concentrations of controlled-release urea each provided nitrogen to rice plants continuously throughout the growing season, thus in generally greater amounts than that of standard urea. Our most concentrated controlled-release treatments may have signaled higher levels of continuous gene expression for products involving nitrogen metabolism compared with the standard urea.

While expression levels increased for the genes *OsNIA*, *OsGS1*, *OsGS2*, *OsGLN1.1*, and *OsGLN1.2*, especially in plants with the stronger controlled-release urea treatments, the

https://doi.org/10.1021/acsomega.3c02113
ACS Omega 2023, 8, 23772−23781

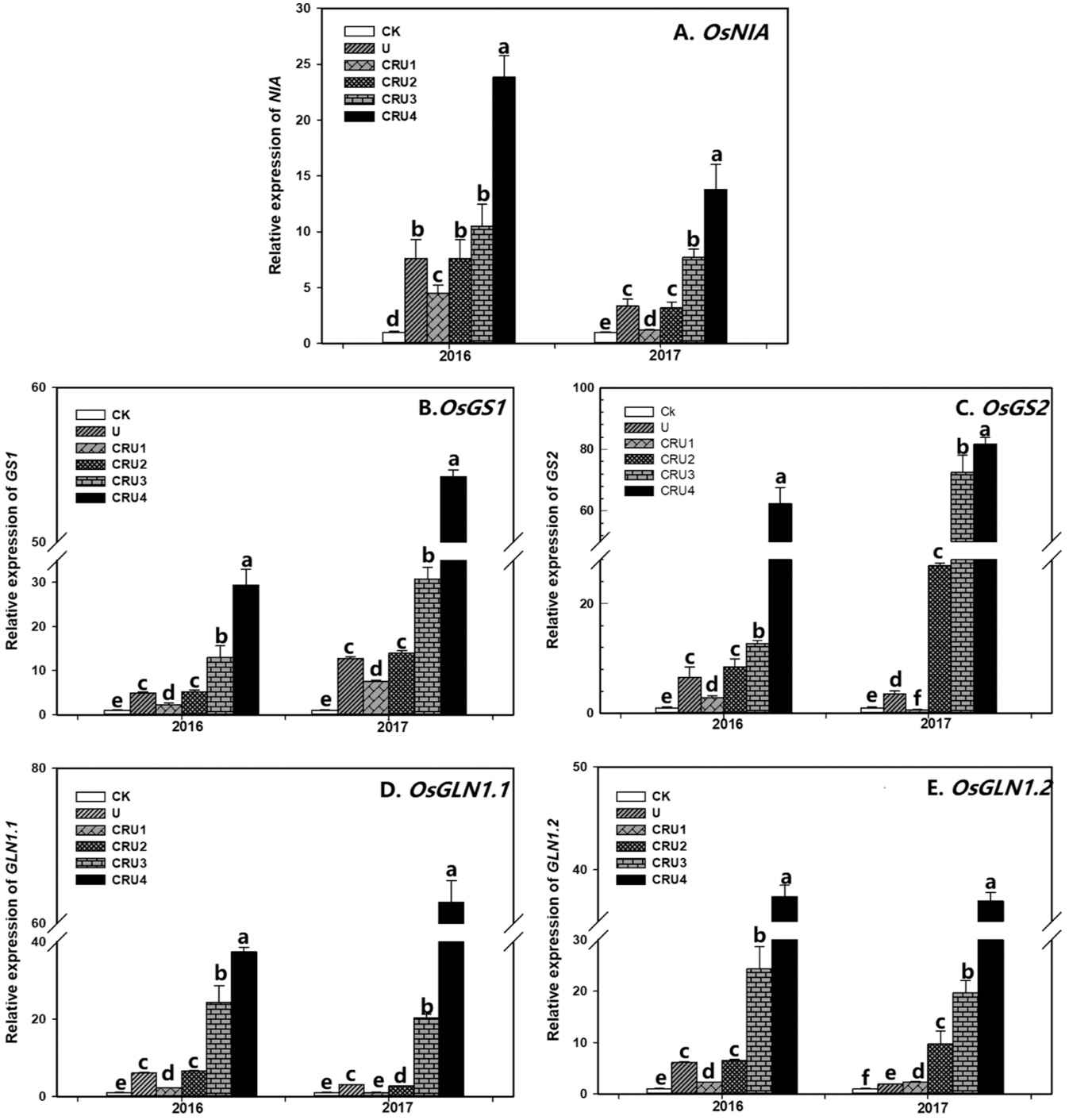

Figure 4. Expression of gene products in the leaf tips of rice at the milk stage during 2016−2017. Gene products were (A) NR *OsNIA*, (B) GS *OsGS*1, (C) GS *OsGS*2, (D) GOGAT *OsGLN*1.1, and (E) GOGAT *OsGLN*1.2. The fertilizer treatments included no applied nitrogen control, CK; standard urea at 360 kg of N/ha U; and treatments involving fertilization with controlled-release urea at 120, 180, 240, and 360 kg of N/ha CRU1, CRU2, CRU3, and CRU4, respectively.

amounts of NR, GS, and GOGAT also increased. Hence, the plants were able to take up more soil nitrogen than corresponding plants fertilized with less or with different fertilizers. Activities of the foregoing genes appeared to be higher in the most well-fertilized, controlled-release urea treatments, resulting in better use of available nitrogen and increased synthesis of enzymes and proteins. Previously, there was a lack of knowledge about the effects of providing different concentrations of controlled-release urea on the physiology and molecular biology of rice under field conditions. Hence, we studied relationships between concentrations of inorganic nitrogen applied to rice roots, the expression of genes involved in nitrogen metabolism, and the levels of resulting enzymes and protein. Pairs of these variables generally correlated with each other, although a notable exception was GOGAT enzyme activity. Compared with the other treatments, our stronger controlled-release urea applications improved soil nitrogen concentrations within rice root zones, resulting in greater nitrogen uptake, which enhanced the expression of genes related to nitrogen metabolism. This in turn led to enhanced

https://doi.org/10.1021/acsomega.3c02113
ACS Omega 2023, 8, 23772−23781

synthesis, transportation, and use of enzymes, which supported further increases in the uptake and use of nitrogen.

Effects of Controlled-Release Urea on Nitrogen Use Efficiency and Yield. Increasing levels of nitrogen fertilization have been found to increase rice biomasses and grain yields (Figure S2).[36,37] Larger overall rice biomasses are needed in part to produce more of the valuable subset, grain yields.[20,38,39] Biomasses and grain yields tend to positively correlate with variables such as plant height and chlorophyll density. We found that the stronger concentrations of controlled-release urea improved rice plant heights and chlorophyll densities, compared with the other treatments including standard urea (Tables S1 and S2). These factors also may have contributed to increases in nitrogen use efficiency, amount of organic matter, and grain yield. Controlled-release urea also provides rice plants with a more persistent nitrogen source, which is not as easily lost through nonuse and leaching compared with standard urea.[21−23] Hence, controlled-release urea often improves nitrogen use efficiencies compared with standard nitrogen fertilizers. Plant nitrogen use efficiency is a complex trait determined by quantitative-trait gene loci and the environment.[13,22] Maintaining good nitrogen use efficiency in crop plants is crucial for sustainable agriculture and increasing grain yields. The natural supply of soil nitrogen is highly variable and strongly affects plant growth and crop yields. Unraveling molecular bases for how plants "sense" and respond to changes in nitrogen availability should help in developing new strategies for increasing nitrogen use efficiencies. Previously, a lack of field study was performed on how providing controlled-release urea to direct-seeded rice affected its nitrogen use efficiency, physiology, molecular biology, and grain yield. The present study found strong correlations among most of these factors. This suggested that compared with the other treatments, our higher concentrations of controlled-release urea first improved root-zone nitrogen concentrations, which promoted higher rates of nitrogen uptake, which led to enhanced expression of genes controlling nitrogen metabolism. These genes indirectly increased the production, transport, and use of nitrogen-synthesizing enzymes and proteins. In turn, the result was added uptake and use of nitrogen and higher nitrogen use efficiencies and grain yields. Hence, controlled-release urea, especially when provided at our two strongest concentrations, can greatly benefit sustainable agriculture while maximizing yields of direct-seeded rice.

CONCLUSIONS

Controlled-release urea improved the N concentration in water and soil compared with standard urea and treatments receiving no N fertilizer. This increased the expression of genes related to nitrogen metabolism including *OsNIA*, *OsGS1*, *OsGS2*, *OsGLN1.1*, and *OsGLN1.2*. The activity of enzymes in rice leaves related to nitrogen metabolism also increased including NR, GS, and GOGAT; hence, protein content was also improved. Strong correlations usually occurred between the levels of inorganic nitrogen found in rice root zones and indices of nitrogen use efficiency, physiology, molecular biology, and grain yield. Controlled-release urea provided at our two highest concentrations also enhanced the expression of genes related to nitrogen metabolism. These benefits helped the rice plants improve their absorption, transport, and use of nitrogen. The hypothesis was that controlled-release urea increases gene transcription, functional enzyme synthesis, and nitrogen use efficiencies of direct-seeded rice, thereby improving its production. Providing controlled-release urea at our two strongest concentrations during seeding can therefore promote sustainable agriculture while maximizing rice yields.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c02113.

Location of field plots, views of field plots and view of rice plants during the milk stages, rice plant heights (cm) measured at different growth stages during 2016, and chlorophyll density (SPAD values) for rice at different growth stages (PDF)

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https://doi.org/10.1021/acsomega.3c02113
ACS Omega 2023, 8, 23772−23781

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was funded by the Young Elite Scientists Sponsorship Program by CAST (2021QNRC001), Shandong Agricultural Innovation Team (SDAIT-2021-04), National Natural Science Foundation of China (grant no. 32202612), and Natural Science Foundation of Shandong Province (grant no. ZR2022QC051). We also thank Cliff G. Martin for reviewing this manuscript.

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https://doi.org/10.1021/acsomega.3c02113
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