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Zinc and nitrogen synergistic act on root-to-shoot translocation and preferential distribution in rice

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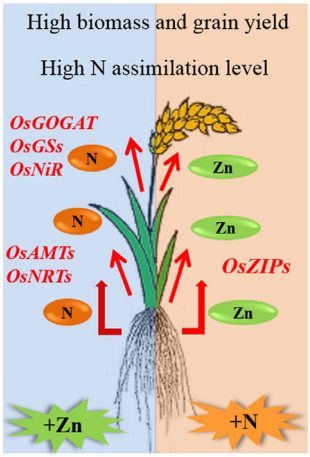
HIGHLIGHTS

- Zn promoted translocation and distribution of N into leaves and brown rice.
- Zn induced the expression levels of N transporter genes in both root and shoot.
- Zn increased the N assimilation level in leaves.
- N promoted translocation and distribution of Zn into leaves and brown rice.
- N up-regulated the expression levels of Zn transporter genes in both root and shoot.

GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: Multiple studies have shown strong relationships between different nutrients in plants, and the important role of N in Zn acquisition and translocation has been recognized.
Objectives: The aim of this study was to estimate the effect of Zn on N uptake, translocation, and distribution in rice as well as the corresponding molecular mechanisms. We also aimed to evaluate the impact of N on the Zn content in rice grains which is closely related to the Zn nutrition in humans with rice-based diets.
Methods: We conducted both field trials and hydroponic cultures of two rice cultivars to analyze the growth and yield, the uptake, translocation, and distribution of N and Zn, as well as the expression of N transport and assimilation genes, and the Zn transporter genes under different combined applications of N and Zn.
Results: Zn supply promoted the root-to-shoot translocation (12–70% increasing) and distribution of N into the leaves (19–49% increasing) and brown rice (6–9% increasing) and increased the rice biomass (by 14–35%) and yield (by 13–63%). Zn supply induced the expression of *OsNRTs* and *OsAMTs* in both roots

Abbreviations: GLY, Guangliangyou 35; Nip, Nipponbare; N, nitrogen; Zn, Zinc; NRT, nitrate transporter; AMT, ammonium transporter; NiR, nitrite reductase; NR, nitrate reductase; GS, glutamine synthetase; GOGAT, glutamate synthase; ZIP, ZRT, IRT-like protein.

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and shoots, but repressed the expression of *OsNiR2*, *OsGS1;2*, and *OsFd-GOGAT* in roots, whereas it activated the expression of *OsNiR2*, *OsGS1;1*, *OsGS2*, and *OsFd-GOGAT* in the shoots. Moreover, the enzyme activities of nitrite reductase, nitrate reductase, and glutamine synthetase increased and the free NO_3^- concentration decreased, but the soluble protein concentration increased significantly in the shoots after Zn supply. Synergistically, N significantly facilitated the root-to-shoot translocation (1.68–11.66 fold) and distribution of Zn into the leaves (1.68–6.37 fold) and brown rice (7–12% increasing) and upregulated the expression levels of Zn transporter genes in both the roots and shoots.

Conclusions: We propose a working model of the cross-talk between Zn and N in rice plants, which will aid in the appropriate combined application of Zn and N fertilizers in the field to improve both N utilization in plants and Zn nutrition in humans with rice-based diets.

Introduction

Nitrogen (N) is a key essential macronutrient that affects plant growth and development and is often a major limiting factor in crop yield production [1,2]. Plants uptake and transport both nitrate (NO_3^-) and ammonium (NH_4^+) through nitrate and ammonium transporters (NRT and AMT) [3]. In plants, nitrate reductase (NR) and nitrite reductase (NiR) reduce NO_3^- to NH_4^+, and NH_4^+ is assimilated into amino acids through the glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle [1,4]. In the last few decades, large amounts of N fertilizers have been applied to arable land in China. Because of the relatively low N use efficiency in crops, excessive N leaching and emissions from farmland cause several environmental problems, such as eutrophication and deterioration of water quality [5–7]. Increasing crop N use efficiency is still an outstanding issue in China and is meaningful for both agricultural production and environmental protection.

Zinc (Zn), an essential microelement required for plant growth, plays both structural and catalytic roles in numerous enzymes that participate in various important metabolic and regulatory processes [8–11]. In most crops, the typical requirement of Zn for adequate growth is 15–20 mg/kg dry weight [12]. When Zn is deficient, plants develop specific symptoms characterized by leaf chlorosis, leaf size reduction, internode shortening, and root apex necrosis, ultimately causing growth suppression and yield reduction [9,10]. Zn deficiency is the most widespread micronutrient disorder in rice plants, affecting up to 50% of the soil in lowland rice production globally [13]. Zn is also often deficient in human populations with rice-based diets [14]. Application of Zn fertilizers to the field is a simple and easy way for farmers to avoid Zn deficiency in crops and simultaneously obtain large grains with high Zn content for human nutrition. Both foliar- and soil-applied Zn significantly increased the grain Zn content in wheat [15].

In a number of physiological studies, strong relationships have been found between different nutrients, and there is evidence that changing one or more nutrients in the growth medium may affect the concentrations of many other nutrients in plants. In recent studies, it has been reported that molybdenum (Mo), potassium (K), and phosphorus (P) can interact synergistically with N in plants. Mo plays a key role in both N uptake and assimilation in winter wheat. The contents of nitrite, ammonium, amino acids, and proteins were significantly increased by the application of Mo [16,17]. In rice, the supply of K increased N use efficiency by promoting the transformation of storage N to photosynthetic N in the leaves [18]. Interestingly, the phosphate starvation response is strongly and actively controlled by N provision, and PHR1, PHO2, and NRT1.1 are the major components that integrate N and P signals in *Arabidopsis*, rice, and wheat [19]. In addition, Zn has the extravagant potential to mitigate the heavy metal toxicity in various plants. For example, the application of zinc oxide nanoparticles significantly diminished cadmium (Cd) and arsenic (As) concentrations in rice and soybean, respectively [20,21], and the application of zinc lysine considerably decreased chromium (Cr) content in spinach [22]. Zn can also mitigate the adverse effects of salt stress in *Brassica juncea* through osmotic adjustment and modulating the oxidative defense system and flavonoid content [23,24].

Kutman et al. [25,26] have previously reported that N nutrition is an important factor in both the acquisition and grain allocation of Zn and Fe in wheat, and that high N supply enhanced the Zn and Fe uptake by up to four fold. However, it has yet to be shown how Zn application affects N uptake, translocation, and distribution in rice plants, and which molecular integrators are mainly involved in the interaction between Zn and N. In this study, we analyzed the rice growth and yield production, N and Zn uptake, translocation, and distribution, as well as the N- and Zn-related gene expression levels, enzyme activities, and levels of metabolites under different combined applications of N and Zn. Our results revealed a clearly synergistic effect between Zn and N mainly on the root-to-shoot translocation and preferential distribution through upregulating the expression levels of N and Zn transporter genes in rice plants. Zn supply also promoted the N assimilation level in rice leaves, thereafter increased the biomass and yield production. Additionally, N supply increased the Zn distribution rate in brown rice. These results provides a theoretical basis for optimizing the application of N and Zn fertilizers in rice cultivation to improve both N use efficiency and Zn nutrition in humans with rice-based diets.

Materials and methods

Plant materials and growth conditions

Two rice cultivars Guangliangyou 35 (GLY) and Nipponbare (Nip) were used in this study. GLY is one of the most widely planted hybrid indica rice cultivars in Hubei Province, China, and Nip is a conventional japonica rice cultivar. Additionally, five different rice cultivars (LYPJ, GD194, 9311, 7954 and ZH11) and *OsGS2* co-suppressed transgenic plants (*osgs2*) [27] with the wild type (Zhonghua 11) were also used for the determination of N and Zn levels.

In the field trial, seeds of GLY and Nip were soaked in sand and grown in a greenhouse at 25–30 °C under natural light. After 20 days, the seedlings were transferred to the field with different combinations of Zn (0 and 30 kg/hm^2) and N (90, 180 and 270 kg/hm^2) supply. In total, six treatments and three biological replicates with completely randomized block designs were set up in the field. For each replicate, ten plants were randomly selected and used for subsequent analysis. At the mature stage, grains and shoot samples were harvested for yield and biomass evaluation, and different organs, including the stem, sheath, flag leaf, other

leaves and brown rice, were sampled to determine the levls of Zn and N.

For hydroponic culture, seeds were soaked in water at 30 °C in the dark for 2 days and then transferred to a net floating on a $CaCl_2$ solution (0.5 mM). After 5 days, the seedlings were transferred to a Yoshida solution (pH 5.6) [28] containing different combinations of Zn (0, 0.012, 0.12, 1.2, and 12 μM) and N (0, 0.288, 2.88, and 14.4 mM) supply, and grown in a greenhouse at 25 °C to 30 °C under natural light. Solutions were renewed every 2 days. In total, 20 treatments and three biological replicates (two or three plants for each replicate in different pots) in a completely random design were set up. Four weeks later, the roots were washed three times with 0.5 mM $CaCl_2$ and separated from the shoots. Different organs, including the shoot basal region (0.5 cm from the root-shoot junction) and different leaves, were sampled to determine the levels of Zn and N. The root and shoot were sampled for the analyses of gene expression levels, enzyme activities, and metabolites, as described below.

Determination of N and Zn levels in plant samples

The harvested samples were dried in an oven at 70 °C for 3 days. After recording the dry weight, the samples were subjected to digestion with 5 ml of 11 N HNO_3 on a heater at up to 150 °C. The concentrations of Zn and N in the digestion solution were determined using an atomic absorption spectrophotometer (WFX-ID) and a flow injection analyzer (FIAstar 5000, Sweden), respectively.

Short-term translocation and distribution of ^{15}N

To analyze the effect of Zn supply on short-term root-to-shoot translocation and distribution of N in different organs, a ^{15}N-labeling experiment was conducted. Seedlings of Nip (5-week-old) grown in the Yoshida solutions with 1.44 mM NH_4NO_3 and 0.012 (−Zn) or 0.12 μM $ZnSO_4$ (+Zn) were exposed to the same solution, but with 1.44 mM $^{15}NH_4^{15}NO_3$. After 24 h, the roots were washed three times with 5 mM $CaCl_2$, and the shoots were separated from the roots. Different organs, including the shoot basal region (0.5 cm from the root-shoot junction) and different leaves, were sampled for ^{15}N determination. The ^{15}N and total N contents were analyzed using an isotope mass spectrometer (ANCA-MS; Europa Scientific, Crewe, UK) and a C/N analyzer (Vario MAX CN; Elementar, Germany), respectively.

Short-term translocation and distribution of ^{67}Zn

To analyze the effect of N supply on short-term root-to-shoot translocation and distribution of Zn in different organs, a ^{67}Zn-labeling experiment was conducted. Seedlings of Nip (5-week-old) grown in the Yoshida solutions with 0.12 μM $ZnSO_4$ and 0.144 mM (-N) or 1.44 mM (+N) NH_4NO_3 were exposed to the same solution, but with 1.2 μM $^{67}ZnSO_4$. After 24 h, the roots were washed three times with 5 mM $CaCl_2$, and the shoots were separated from the roots. Different organs, including shoot basal region (0.5 cm from the root-shoot junction) and different leaves, were sampled for ^{67}Zn determination. The concentrations of ^{67}Zn and ^{66}Zn were determined in isotope mode using ICP-MS (Agilent 7700).

RNA extraction and quantitative real-time PCR analyses

Total RNA was extracted from root and shoot samples using an RNeasy Plant Mini Kit (Yeasen) and converted to cDNA using Rever Tra Ace qPCR RT Master Mix with gDNA remover (Yeasen) following the manufacturer's protocol. The cDNA was amplified using the SYBR Green Real-Time PCR Master Mix Kit (KAPA) and quantitative real-time PCR was performed using a Quant Studio TM 6 Flex System (Applied Biosystems, Foster City, CA, USA) with specific gene primers for *OsNRTs*, *OsAMTs*, *OsNAR*, *OsNiR2*, *OsGS1;1*, *OsGS1;2*, *OsGS2*, *OsFd-GOGAT*, and *OsZIPs* (Supplementary Table S1). The relative gene expression levels were normalized using an internal standard (*OsUbiquitin*) and calculated using the $2^{-\Delta\Delta Ct}$ method with CFX Manager Software (Bio-Rad).

Enzyme activity analyses

The enzyme activities of NR (EC 1.7.1.3), NiR (EC 1.7.2.1), and GS (EC 6.3.1.2) were analyzed based on previously described methods [29–31]. Fresh root and shoot samples were ground and homogenized in the extraction buffer on ice. The determination of NR activity in the supernatant was based on the formation of nitrite in the reaction medium. To terminate the reaction, 1 ml of 1% sulfanilamide and 1 ml of 0.02% naphthyl ethylenediamine-HCl were added. NiR activity was assayed by the reduction of NO_2^- in the assay mixture. To start the reaction, 100 ml of 0.12 M $Na_2S_2O_4$ dissolved in 0.2 M $NaHCO_3$ was added. After incubation at 30 °C for 60 min, the reaction was terminated by vigorous vortexing until the color of the methyl viologen disappeared completely. The GS activity in the supernatant was measured in a pre-incubated assay buffer at 37 °C. The reaction was terminated after 30 min by adding an acidic $FeCl_3$ solution. After allowing 10 min for the color to develop, the absorbance of the supernatant was measured using a spectrophotometric quantification reader at 540 nm.

N metabolite determination

The concentrations of free NH_4^+, NO_3^- and soluble proteins were determined according to the methods described by Cai et al. [31]. Fresh root and shoot samples were homogenized by grinding on ice with an extraction buffer. The free NH_4^+ and NO_3^- in the supernatant were determined by the Berthelot color reaction method and the Griess method, respectively. Soluble protein concentration was measured using the Bradford method.

Results

Different responses of GLY and Nip to N or Zn supply

GLY is one of the most widely planted hybrid indica rice cultivars in Hubei Province, China, and Nip is a conventional japonica rice cultivar. Herein, we evaluated the biomass production and N or Zn accumulation in GLY and Nip grown hydroponically in solutions with different N or Zn supplies. Root and shoot dry weights were higher and the accumulation of N and Zn was greater in the GLY cultivar than the conventional Nip cultivar (Supplementary Figs. S1 and S2). Both the root and shoot dry weights of GLY increased by 31.02% and 40.73%, respectively, with N supply from 0.24 to 0.48 mM, held steady with N supply from 0.48 to 8.64 mM, and had another significant increase (by 35.58% and 27.46%, respectively) with N supply from 8.64 to 14.4 mM (Supplementary Fig. S1A, S1C, S1D). The results were quite different in Nip cultivar, in which both root and shoot dry weights increased gradually with N supply from 0.24 to 2.88 mM and then held steady with N supply from 2.88 to 14.4 mM (Supplementary Fig. S1B, S1C, S1D). Interestingly, the N accumulation in both the roots and shoots of GLY and Nip was the same under different N supply conditions ranging from 0.24 to 14.4 mM (Supplementary Fig. S1E, S1F). Additionally, very similar results were also observed in both root and shoot dry weights of GLY and Nip grown under different Zn supply conditions ranging from 0 to 6.0 μM Zn (Supplementary Fig. S2A-S2D).

However, the Zn accumulations in both the roots and shoots of GLY and Nip were different from the results above and increased gradually with increasing Zn supply (Supplementary Fig. S2E, S2F). These results clearly show that GLY and Nip have different responses to N or Zn supply: GLY grew much better under high N or Zn supply conditions, whereas Nip performed better in moderate N or Zn supply conditions.

Zn promotes rice growth and yield production under N supply conditions

To assess the effect of Zn on plant growth and yield production in rice, GLY and Nip were grown in both solutions and soils with different combinations of N and Zn supply. The results for GLY and Nip were quite similar, except for the root dry weight of rice grown hydroponically in solutions. At 0 mM N, both plant height and shoot dry weight did not change appreciably with increasing Zn supply, except that at 1.2 μM Zn, plant height increased significantly (Supplementary Fig. S3A-D; Fig. S4A and S4B). At 0.288 mM N, both plant height and shoot dry weight increased gradually with Zn supply from 0 to 0.12 μM, and decreased with Zn supply from 0.12 to 12 μM (Supplementary Fig. S3A-D; S4A and S4B). At 2.88 and 14.4 mM N, both plant height and shoot dry weight increased gradually with Zn supply from 0 to 0.12 μM, and held steady with higher Zn supply (Supplementary Fig. S3A-D; S4A and S4B). However, the results for root length were significantly different than those for plant height. At 0 mM N, the root length increased gradually with Zn supply from 0 to 0.12 μM, and held steady with higher Zn supply; whereas at 0.288 and 2.88 mM N, root length did not change appreciably with increasing Zn supply. At higher N supply (14.4 mM), root length decreased gradually with increasing Zn supply (Supplementary Fig. S3E, S3F). In addition, the root dry weight was quite different between GLY and Nip, except that there were no significant changes with increasing Zn supply in either GLY or Nip at 0 mM N (Supplementary Fig. S4C, S4D). The highest root dry weight was observed at 0.12 μM Zn in GLY under each N supply condition. In contrast, in Nip, the best results for root dry weight were obtained with 0.012 μM Zn at 0.288 mM N, and this did not change significantly with increasing Zn supply at either 2.88 or 14.4 mM N (Supplementary Fig. S4C, S4D).

Furthermore, similar results were observed for both GLY and Nip in the field trial. At 90 kg/hm^2 N supply, no obvious changes were found in either yield or straw dry weight after 30 kg/hm^2 Zn supply, whereas higher yield and straw dry weight were observed after 30 kg/hm^2 Zn supply under both 180 and 270 kg/hm^2 N supply conditions (Fig. 1). At 180 kg/hm^2 N supply, the yield increased by 20.93% and 63.30% in GLY and Nip, respectively, and the straw dry weight increased by 35.26% and 15.51% in GLY and Nip, respectively, after 30 kg/hm^2 Zn supply (Fig. 1). At 270 kg/hm^2 N supply, the yield increased by 13.34% and 38.04% in GLY and Nip, respectively, and the straw dry weight increased by 14.52% and 14.30% in GLY and Nip, respectively, after 30 kg/hm^2 Zn supply (Fig. 1).

Zn promotes root-to-shoot translocation and preferential distribution of N in rice

To investigate the physiological mechanism of the phenotype observed above, we determined the root and shoot concentrations of N, and calculated the N content, root uptake ability, root-to-shoot translocation and distribution in GLY and Nip under different combinations of N and Zn supply. At 0 mM N, the N concentrations in both the roots and shoots of GLY and Nip did not change much with increasing Zn supply, except that slight decreases were exhibited in the N concentrations of the roots and shoots in Nip with a 12.0 μM Zn supply (Supplementary Fig. S5A-D). Significant decreases in N concentrations in both the roots and shoots of GLY and Nip were observed with Zn supply under the other N supply conditions, although no significant change was displayed in the N concentration of the roots in GLY under 14.4 mM N supply conditions (Supplementary Fig. S5A-D). Although the root uptake ability of N was lower under high Zn supply conditions (2.88 mM N), no obvious changes were observed in the root uptake ability of N in both GLY and Nip under other different combinations of N and Zn supply (Supplementary Fig. S5G, S5H). The N content in the whole plant did not change significantly with increasing Zn supply (Supplementary Fig. S5E, S5F).

The root-to-shoot translocation of N was completely different from the results of the N concentration shown above. The root-to-shoot translocation of N in both GLY and Nip clearly increased after Zn supply under each N supply condition (Fig. 2A, 2B). After Zn supply, increases of 0.89–12.16% and 34.16–53.99% at 0 mM N, 3.91–24.33% and 1.74–35.27% at 0.288 mM N, 6.77–23.09% and 15.46–69.87% at 2.88 mM N, and 10.40–28.75% and 12.75–26.98% at 14.4 mM N, were observed in GLY and Nip, respectively (Fig. 2A, 2B). The results of measuring N distribution to different organs clearly showed significant decreases in N distribution in the roots as well as significant increases in N distribution in the shoots, including basal nodes and leaves, after Zn supply under each N supply condition (Fig. 2C, 2D). After Zn supply, the N distribution in the shoots increased by 3.47–19.36% and 3.79–18.54% at 0 mM N, 15.20–37.03% and 4.88–14.49% at 0.288 mM N, 9.77–24.97% and 15.11–49.09% at 2.88 mM N, and 10.87–28.10% and 10.95–20.22% at 14.4 mM N in GLY and Nip, respectively (Fig. 2C, 2D).

To confirm these results, we performed a short-term (24 h) labeling experiment with a stable isotope ^{15}N in Nip. Significant increases in both root-to-shoot translocation of ^{15}N and distribution in the leaves were observed under the +Zn condition (Fig. 3). The root-to-shoot translocation of ^{15}N increased by 7.53%, whereas the ^{15}N distribution in the leaves increased by 10.65% after Zn supply (Fig. 3).

In the field trial, more N was distributed into brown rice after 30 kg/hm^2 Zn supply at both 180 and 270 kg/hm^2 N supply conditions. The N distribution increased from 74% to 80% and 72% to 81% at 180 and 270 kg/hm^2 N conditions, respectively, after 30 kg/hm^2 Zn supply in GLY, and N distribution increased from 54% to 62% and 51% to 57% at 180 and 270 kg/hm^2 N conditions, respectively, after 30 kg/hm^2 Zn supply in Nip (Supplementary Fig. S6).

N promotes root-to-shoot translocation and preferential distribution of Zn in rice

To test the effect of N supply on Zn in rice plants, we determined the root and shoot concentrations of Zn and calculated the content, root uptake ability, root-to-shoot translocation, and distribution of Zn in different organs of GLY and Nip under different combinations of N and Zn supply. Results clearly showed that the supply of N significantly decreased Zn concentrations of the roots and shoots as well as the root uptake ability of Zn in both GLY and Nip, especially under $\geq$ 0.12 μM Zn supply conditions (Supplementary Fig. S7A-D, S7G and S7H). The Zn content in the whole plant was quite different: the Zn content dramatically increased after 0.288 mM N supply, but decreased and remained stable with higher N supply (Supplementary Fig. S7E, S7F). However, significant increases in root-to-shoot translocation of Zn were observed after N supply in both GLY and Nip under each Zn supply condition (Fig. 4). After N supply, root-to-shoot translocation of Zn increased 2.68–2.84 and 2.26–4.51 fold at 0 μM Zn, 2.48–3.24 and 2.99–3.25 fold at 0.012 μM Zn, 4.21–4.88 and 10.40–11.66 fold at 0.12 μM Zn, 1.56–3.18 and 3.13–5.34 fold at 1.2 μM Zn, 1.59–2.00 and 1.21–1.68 fold at 12 μM Zn, in GLY and Nip, respectively (Fig. 4A, 4B).

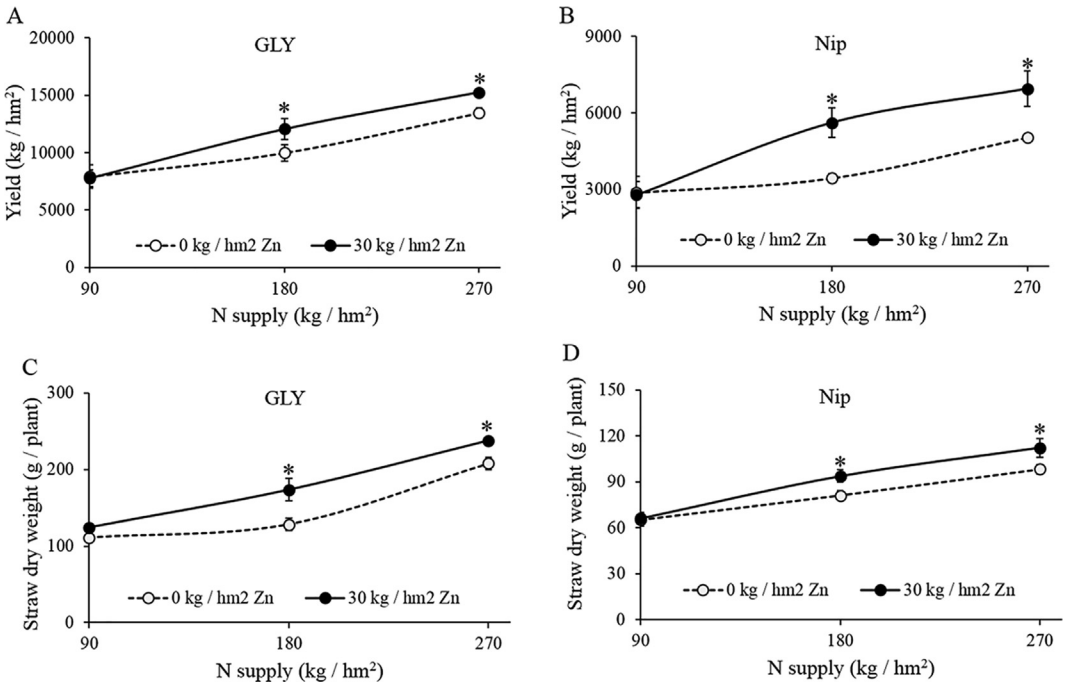

Fig. 1. Grain yield (A, B) and straw dry weight (C, D) of GLY and Nip grown in the field with different combinations of N and Zn supply until mature stage. Data are means ± SD of three biological replicates. * indicates significant difference at $P < 0.05$ by Tukey's test.

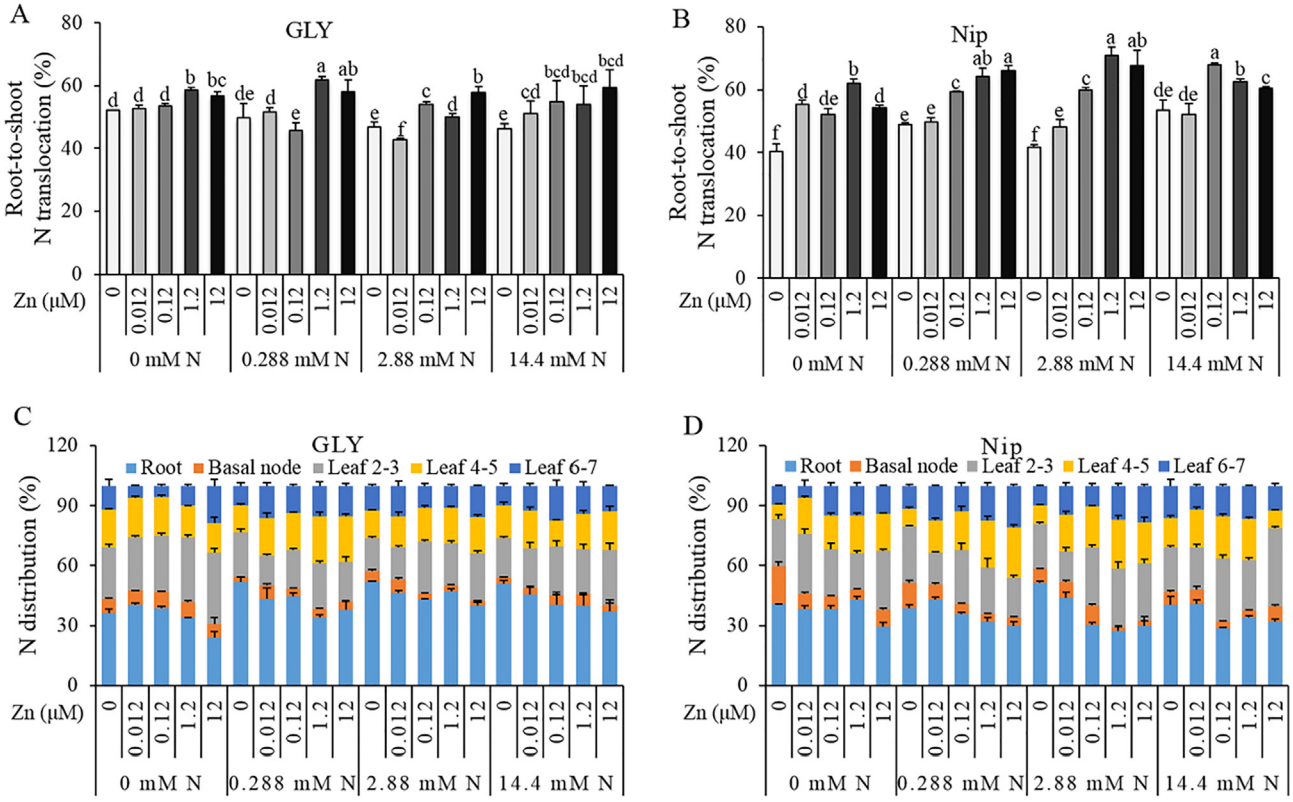

Fig. 2. Root-to-shoot translocation (A, B) and distribution (C, D) of N in GLY and Nip grown in Yoshida solutions with different combinations of N and Zn supply for 4 weeks. Data are means ± SD of three biological replicates. Different letters indicate significant difference at $P < 0.05$ by Tukey's test.

Measurements of Zn distribution in different organs clearly showed that the Zn distribution in the roots significantly decreased, whereas the Zn distribution in the shoots (including basal nodes and leaves), especially in the leaves, significantly increased after N supply under each Zn supply condition (Fig. 4C, 4D). After N supply, Zn distribution in the shoots increased 1.73–1.88 and 1.27–2.40 fold at 0 μM Zn, 1.60–1.83 and 1.65–1.86 fold at 0.012 μM Zn, 3.09–3.18 and 5.63–6.37 fold at 0.12 μM Zn, 1.48–2.06 and 1.77–2.25 fold

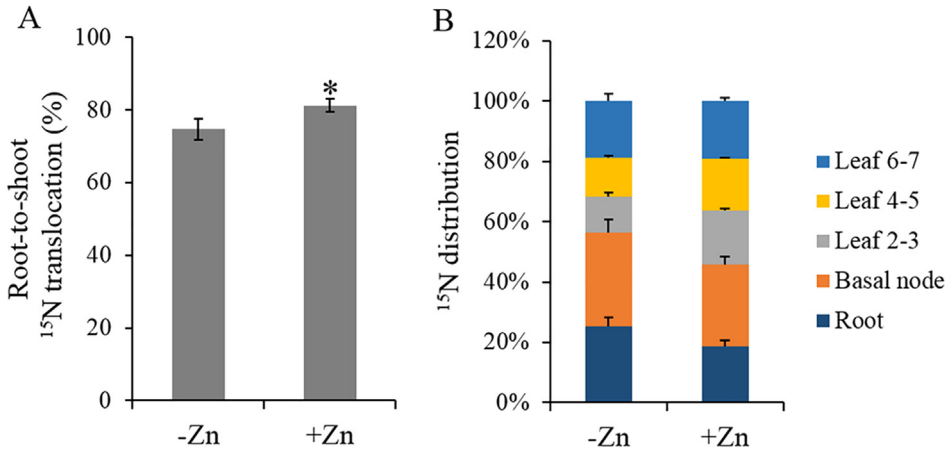

Fig. 3. Root-to-shoot translocation (A) and distribution (B) of ^{15}N in Nip grown in Yoshida solutions containing 1.44 mM $^{15}NH_4^{15}NO_3$ for 24 h under –Zn (0.012 μM) and +Zn (0.12 μM) conditions. Data are means ± SD of three biological replicates. * indicates significant difference at $P < 0.05$ by Tukey's test.

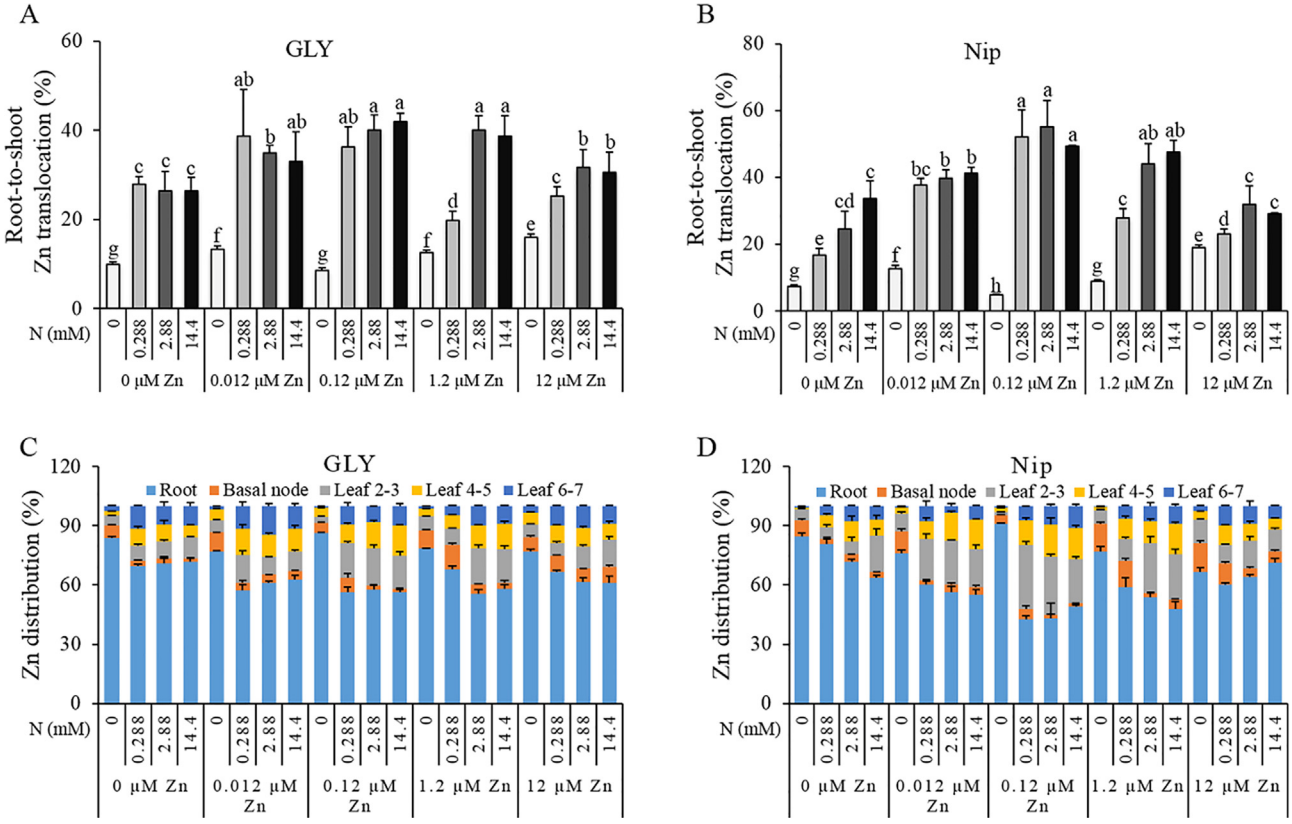

Fig. 4. Root-to-shoot translocation (A, B) and distribution (C, D) of Zn in GLY and Nip grown in Yoshida solutions with different combinations of N and Zn supply for 4 weeks. Data are means ± SD of three biological replicates. Different letters indicate significant difference at $P < 0.05$ by Tukey's test.

at 1.2 μM Zn, and 1.44–1.68 and 1.08–1.20 fold at 12 μM Zn, in GLY and Nip, respectively (Fig. 4C, 4D).

To confirm these results, we performed a short-term (24 h) labeling experiment with the stable isotope ^{67}Zn in Nip. Significant increases in both root-to-shoot translocation of ^{67}Zn and distribution in the leaves were observed under the +N condition (Fig. 5). The root-to-shoot translocation of ^{67}Zn increased by 21.29%, while the ^{67}Zn distribution in the leaves increased by 29.23% after N supply (Fig. 5).

In addition, we analyzed the root-to-shoot translocation and distribution of N and Zn in another five different rice cultivars (LYPJ, GD194, 9311, 7954, and ZH11) at both the tillering and heading stages under different N supply conditions. Similar results were observed. Both the root-to-shoot translocation and distribution of N in the shoots, including the stems and leaves, gradually increased with the increasing N supply in all five rice cultivars at the two developmental stages (Supplementary Fig. S8A-D). Synergistically, the root-to-shoot translocation and distribution of Zn in the shoots also gradually increased with the increasing N supply in all five rice cultivars at two developmental stages (Supplementary Fig. S8E-H). The increase in both root-to-shoot translocation and distribution of N and Zn in all five rice cultivars was more pronounced at the tillering stage than at the heading stage (Supplementary Fig. S8).

The *OsGS2* co-suppressed transgenic plants (*osgs2*) and wild-type plant (WT) were also used to further confirm these results.

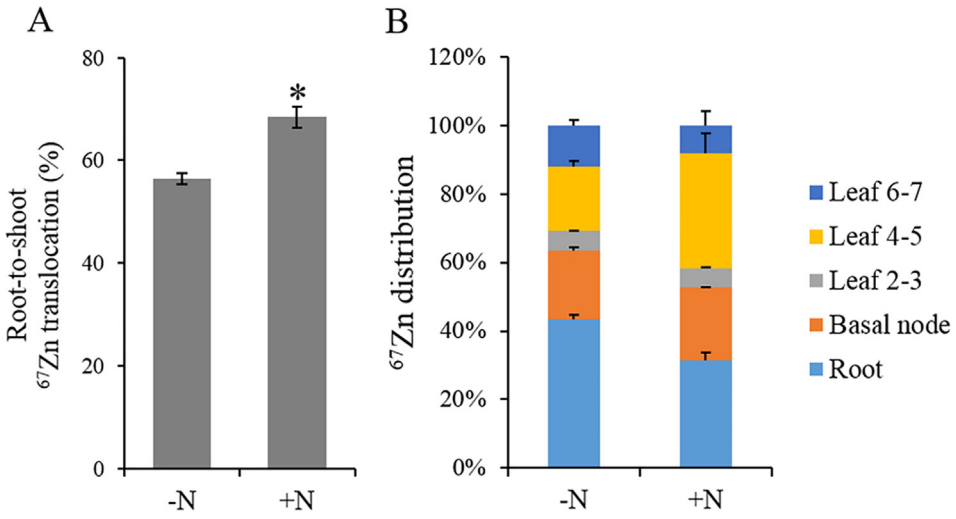

Fig. 5. Root-to-shoot translocation (A) and distribution (B) of ^{67}Zn in Nip grown in Yoshida solutions containing 1.2 μM $^{67}ZnSO_4$ for 24 h under –N (0.288 mM) and +N (2.88 mM) conditions. Data are means ± SD of three biological replicates. * indicates significant difference at P < 0.05 by Tukey's test.

Compared with WT (23%), *osgs2* had a lower N distribution (17%) in the new leaves (Supplementary Fig. S9A). Synchronously, a lower Zn distribution in the new leaves was observed in *osgs2* (11%) than in WT (15%) (Supplementary Fig. S9B).

In the field trial, Zn distribution in brown rice was increased with higher N supply at both 0 and 30 kg/hm^2 Zn conditions. Compared with the 180 kg/hm^2 N supply condition, the Zn distribution increased by 9% and 12% in GLY at 270 kg/hm^2 N supply under 0 and 30 kg/hm^2 Zn conditions, respectively; the corresponding increase in Nip was 7% and 10% in Nip at 270 kg/hm^2 N supply under 0 and 30 kg/hm^2 Zn conditions, respectively (Supplementary Fig. S10).

Effect of Zn on the expression levels of N transport and assimilation genes

To understand the molecular mechanism of the effect of Zn supply on N uptake and translocation, we analyzed the expression levels of N transport and assimilation genes in both the roots and shoots of Nip under different combinations of N and Zn supply. The results showed that the expression levels of low-affinity nitrate transporter genes *OsNRT1.1A* and *OsNRT1.1B* were higher under + N conditions, and the high-affinity nitrate transporter genes *OsNAR2.1* and *OsNRT2.2* were dramatically induced under -N conditions (Fig. 6). Interestingly, the expression levels of both nitrate (*OsNRT1.1A*, *OsNRT1.1B*, *OsNAR2.1*, and *OsNRT2.2*) and ammonium (*OsAMT1;1*, *OsAMT1;2*, *OsAMT1;3*, *OsAMT2;3*, *OsAMT3;2*, and *OsAMT3;3*) transporter genes were upregulated by Zn supply in the roots (Fig. 6). However, the expression levels of the nitrite reductase gene (*OsNiR2*) and ammonium assimilation genes (*OsGS1;2* and *OsFd-GOGAT*) were downregulated by Zn supply in the roots (Fig. 6). In contrast, the expression levels of the nitrite reductase gene (*OsNiR2*) and ammonium assimilation genes (*OsGS1;1*, *OsGS2*, and *OsFd-GOGAT*) were upregulated by Zn supply in the shoots (Fig. 6). The nitrate transporter genes (*OsNRT1.1A*, *OsNRT1.1B*, and *OsNRT2.4*) were also upregulated by Zn supply in the shoots (Fig. 6).

Effect of N on the expression levels of Zn transporter genes

Transporters belonging to the ZIP (ZRT, IRT-like protein) family are thought to be the primary Zn transporters involved in Zn uptake [32]. To understand the molecular mechanism of the effect of N supply on Zn uptake, we analyzed the expression levels of Zn transporter genes, including *OsZIP4*, *OsZIP6*, *OsZIP7*, *OsZIP8*, *OsZIP9*, *OsZIP10*, and *OsZIP11* in the roots and shoots of Nip under different combinations of N and Zn supply. A very similar expression pattern of these Zn transporter genes was observed in the roots and shoots. The expression levels of all these *OsZIPs* significantly increased in roots and shoots after N supply under both -Zn and +Zn conditions, except for *OsZIP11* (Fig. 7).

Effect of Zn on enzyme activities and metabolites involved in N assimilation

To further investigate the effect of Zn on N assimilation, we determined the activities of NR, NiR, and GS, together with the concentrations of free NH_4^+, NO_3^-, and soluble protein in both the roots and shoots of Nip under different combinations of N and Zn supply. The results showed that higher activities of NR and NiR were observed under +N conditions, and +Zn significantly increased the activities of NR, NiR, and GS under both –N (by 39.25%, 12.58% and 24.77%, respectively) and +N (by 37.11%, 8.00% and 61.41%, respectively) conditions in the shoots of Nip, whereas no obvious changes after +Zn treatment were observed in the roots (Fig. 8). The concentration of free NH_4^+ did not change significantly after Zn supply in both the roots and shoots (data not shown); in contrast, the free NO_3^- decreased to 66.10% and 81.77% in the roots and shoots, respectively, under –N condition and decreased to 73.75% and 89.18% in both the roots and shoots, respectively, under +N condition after Zn supply (Fig. 9A, 9B). The soluble protein concentration did not change significantly in the roots after Zn supply but increased to 1.92- and 2.40-fold in the shoots after Zn supply under –N and +N conditions, respectively (Fig. 9C, 9D).

Discussion

Zn and N acted synergistically in rice

Interactions among nutrient elements have been recognized for many years. Previously, Kutman et al. reported that N nutrients play an important role in Zn allocation to grains in wheat [25,26]. However, the physiological and molecular mechanisms of N-Zn interactions are not fully understood. In this study, our results demonstrated a working model of the interaction between N and Zn in rice plants (Fig. 10).

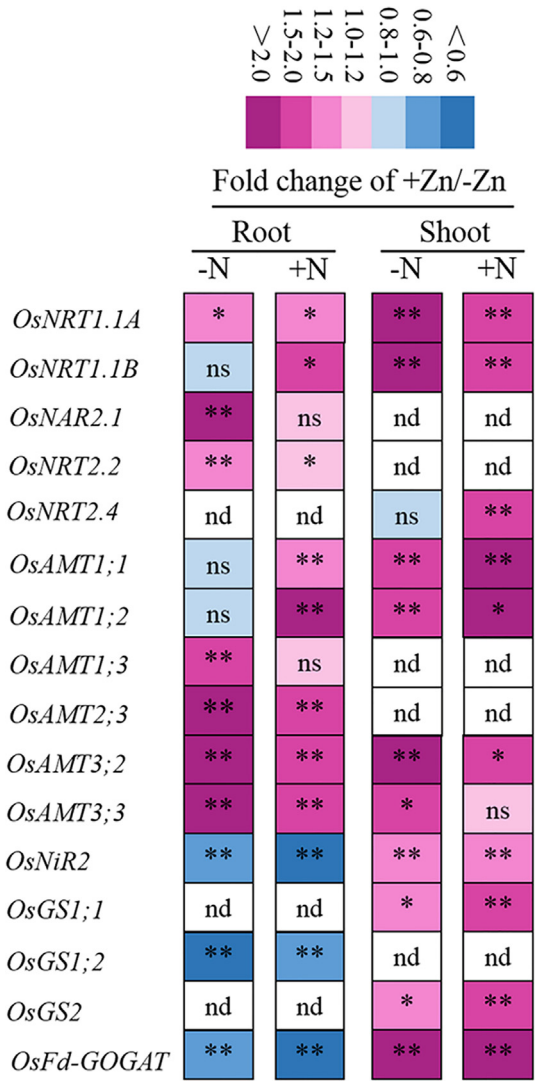

Fig. 6. Expression levels of N transport and assimilation genes in the roots and shoots of Nip grown in Yoshida solutions with different combinations of N and Zn supply for 4 weeks. –Zn: without Zn, +Zn with 0.12 μM Zn, –N: with 0.288 mM N, +N: with 2.88 mM N. Data are fold changes of expression levels of genes under +Zn compared to -Zn conditions. * and ** indicate significant difference at $P < 0.05$ and $P < 0.01$, respectively, by Tukey's test. "ns" means no significance, "nd" means not detected.

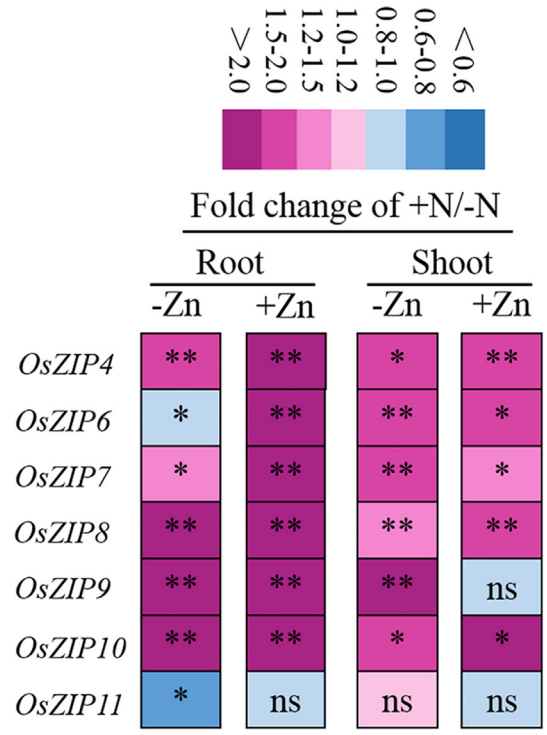

Fig. 7. Expression levels of Zn transporter genes in the roots and shoots of Nip grown in Yoshida solutions with different combinations of N and Zn supply for 4 weeks. –Zn: without Zn, +Zn with 0.12 μM Zn, -N: with 0.288 mM N, +N: with 2.88 mM N. Data are fold changes of expression levels of genes under +N compared to -N conditions. * and ** indicate significant difference at $P < 0.05$ and $P < 0.01$, respectively, by Tukey's test. "ns" means no significance.

Nitrate and ammonium transporter genes (*OsNRTs* and *OsAMTs*) are two major gene families involved in the uptake and distribution of NO_3^- and NH_4^+ in rice plant [33–41]. Our results clearly showed that Zn supply up-regulated the expression levels of *OsNRTs* and *OsAMTs* in both the roots and shoots, which contributed to higher uptake, translocation, and preferential distribution of N into the growth center, including new leaves and spikeletes, ultimately increasing shoot biomass and yield production of rice (Figs. 1–3, 10). After NO_3^- entering into root cells, it can be reduced to NH_4^+ by NR and NiR or translocated into shoot through nitrate transporters (NRTs) [1,42–44]. Thereafter, NH_4^+ can be assimilated into glutamine by GS/GOGAT cycle, which is a key and limiting factor controlling N assimilation from inorganic N into organic N [1,42–46]. In our study, due to more N was translocated and distributed into the leaves after Zn supply, the expression levels of genes involved in nitrate reduction (*OsNiR2*) and ammonium assimilation (*OsGS1;1*, *OsGS2* and *OsFd-GOGAT*) were significantly up-regulated to accelerate the conversion of inorganic N into organic N and produce more amino acids and proteins for plant growth (Figs. 6 and 10). This was further verified by the results of the N metabolites and enzyme activities in our study. The activities of the NR, NiR, and the GS were significantly increased after Zn supply, accompanied by decreased concentration of substrate (free NO_3^-) and increased concentration of product (soluble protein) in the leaves (Figs. 8–10). These results indicated that Zn supply accelerated the N assimilation level in rice leaves, which contributed to the increased biomass and yield production. However, the decreased expression levels of nitrite reductase gene (*OsNiR2*) and ammonium assimilation genes (*OsGS1;2* and *OsFd-GOGAT*) were observed in the roots after Zn supply (Figs. 6 and 10), which may be triggered by the lower concentration of NO_3^- in the roots, as these enzymes are induced by NO_3^- reported previously [47,48].

Synergistically, the application of N also significantly promoted the root-to-shoot translocation and preferential distribution of Zn into new leaves and spikeletes through up-regulating the expression levels of *OsZIPs* genes, including *OsZIP4*, *OsZIP6*, *OsZIP7*, *OsZIP8*, *OsZIP9*, and *OsZIP10* (Figs. 4, 5, 7, 10). Transporters belonging to the ZIP (ZRT, IRT-like protein) family are thought to be the primary Zn transporters involved in Zn uptake [32]. In rice, the functions of *OsZIP4*, *OsZIP6*, *OsZIP7*, *OsZIP8*, and *OsZIP9* were reported to be responsible for the uptake and preferential distribution of Zn to developing tissues, and their expression levels are all induced by Zn deficiency in both the roots and shoots [49–54]. However, the function of *OsZIP10* is less understood yet. Since the plant dry weight increased significantly after N supply, the decreased concentration of Zn in both the roots and shoots under high level of N may influenced by biomass as biomass dilution [55]. Thereafter, the lower Zn concentration caused a Zn deficient signal in plant and induced the expression of *OsZIP4*, *OsZIP6*, *OsZIP7*, *OsZIP8*, and *OsZIP9* in both the roots and shoots to uptake and translocate more Zn to developing tissues for plant growth (Figs. 4, 5, 7, 10).

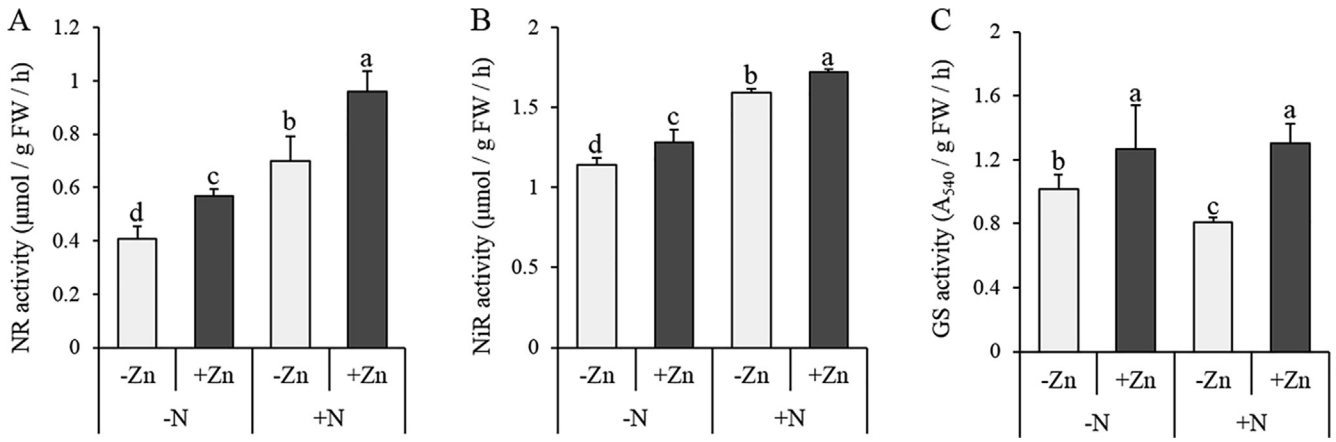

Fig. 8. Activities of enzymes involved in N assimilation in the shoots of Nip grown in Yoshida solutions with different combinations of N and Zn supply for 4 weeks. –Zn: without Zn, +Zn with 0.12 μM Zn, -N: with 0.288 mM N, +N: with 2.88 mM N. Data are means ± SD of three biological replicates. Different letters indicate significant difference at $P < 0.05$ by Tukey's test.

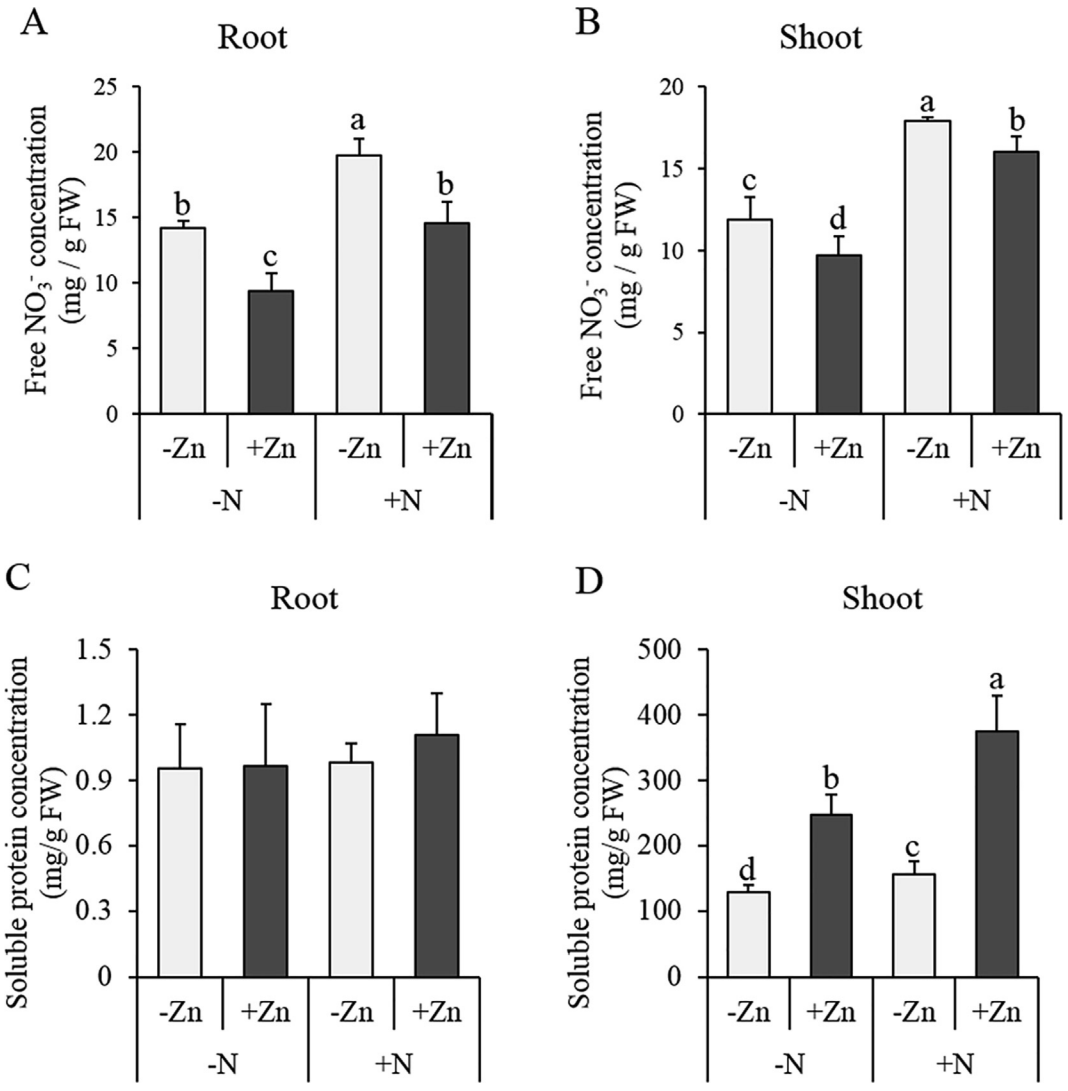

Fig. 9. Concentrations of free NO_3^- and soluble proteins of Nip grown in Yoshida solutions with different combinations of N and Zn supply for 4 weeks. –Zn: without Zn, +Zn with 0.12 μM Zn, -N: with 0.288 mM N, +N: with 2.88 mM N. Data are means ± SD of three biological replicates. Different letters indicate significant difference at $P < 0.05$ by Tukey's test.

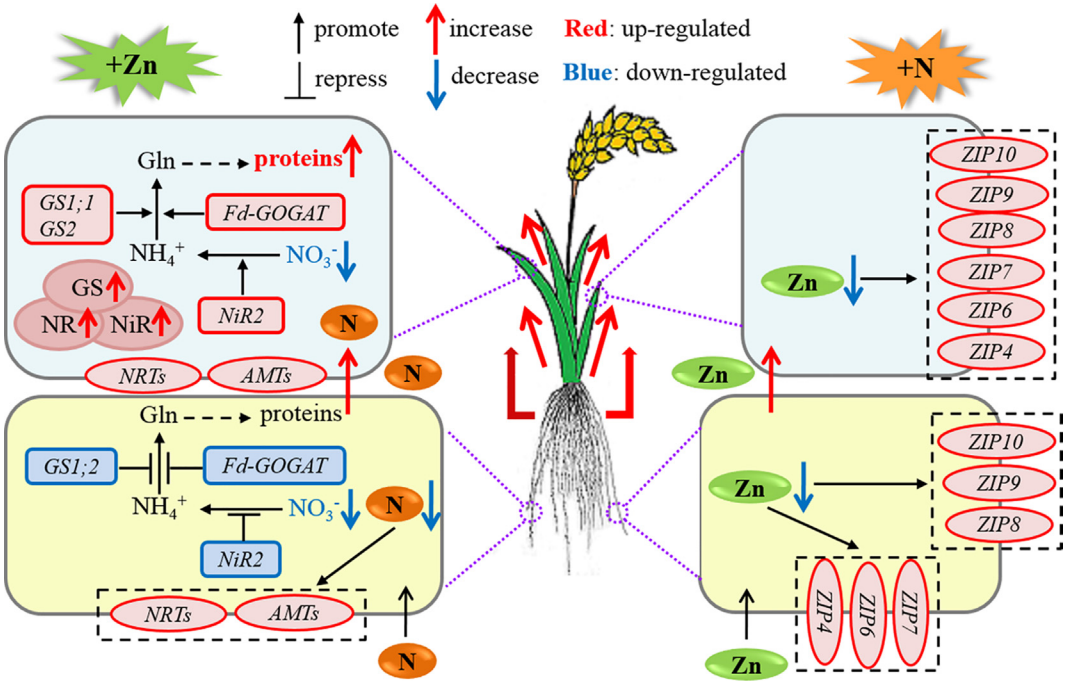

Fig. 10. A working model of the interaction between Zn and N in rice plants.

Physiological and molecular mechanisms of N-Zn interactions are conserved in different rice cultivars

In the present study, we used two different rice cultivars (GLY and Nip) and evaluated the different effects of N-Zn interactions. GLY is one of the most widely planted hybrid indica rice cultivars in Hubei Province, China, and Nip is a conventional japonica rice cultivar. Compared with Nip, GLY had higher biomass, N and Zn accumulation (Supplementary Fig. S1, S2). Considering the different responses to N or Zn supply between GLY and Nip, we speculated that there were genotypic differences in Zn-N interactions between GLY and Nip. Unexpectedly, the physiological and molecular mechanisms of Zn-N interactions were relatively conserved between GLY and Nip, which is quite consistent with the results of N-P interaction reported by Medici et al. and Wang et al. [24,56]. The authors reported that a combination of local and long-distance N signaling actively controlled the P starvation response in *Arabidopsis*, and demonstrated that this phenomenon was also conserved in rice and wheat [24]. Moreover, Wang et al. found that a transcription factor NIGT1.2 could modulated both Pi uptake and N influx during Pi starvation in both *Arabidopsis* and maize [56].

Combined application of Zn and N fertilizers is a good strategy for improving both N utilization in rice and Zn nutrition in humans

Our results clearly showed that Zn significantly promoted the root-to-shoot translocation and distribution of N into the new leaves and grains in rice (Figs. 2 and 3; Supplementary Fig. S6), which suggests that an appropriate supply of Zn fertilizer in low Zn regions is beneficial for improving the N utilization and yield production in crops, thereby reducing the environmental pollution caused by excessive N leaching and emissions from farmland. Conversely, we found that N also significantly increased the root-to-shoot translocation and distribution of Zn into the new leaves, as well as the distribution of Zn in the brown rice (Figs. 4 and 5; Supplementary Fig. S8, S10). This is quite consistent with the results reported by Kutman et al. that N nutrition is a critical factor in grain allocation of Zn in wheat [25,26]. Considering the human health risk from Zn deficiency, the appropriate supply of more N fertilizer is beneficial for increasing Zn concentration in rice grains and can improve Zn nutrition in humans with rice-based diets. Our results provide further evidence that the appropriate combined application of Zn and N fertilizers in the field is advantageous for agricultural production, environmental protection and human health.

Conclusion

In hydroponic culture, Zn supply significantly increased the root-to-shoot translocation and distribution of N into the leaves through upregulating the expression levels of N transporter genes, including *OsNRTs* and *OsAMTs*, in both the roots and shoots. However, slight decreases in N concentration and no significant changes in N content were observed after Zn supply. The application of Zn also induced the expression levels of N assimilation genes (*OsNiR2*, *OsGS1;1*, *OsGS2*, and *OsFd-GOGAT*) and the activities of NR, NiR, and GS in the shoots, but decreased the expression levels of *OsNiR2*, *OsGS1;2*, and *OsFd-GOGAT* in the roots. Therefore, Zn supply promoted the N assimilation level in the rice shoots, which contributed to the higher biomass. Synergistically, the application of N significantly increased the root-to-shoot translocation and distribution of Zn into the leaves through upregulating the expression levels of Zn transporter genes (*OsZIPs*) in both the roots and shoots. Whereas significant decreases in Zn concentration and uptake were observed after N supply. Quite similar results were observed in the field trial that the application of 30 kg/hm^2 Zn significantly increased the straw dry weight, yield production and N distribution rate in the brown rice under both 180 and 270 kg/hm^2 N conditions. Most importantly, higher N supply also increased the distribution rate of Zn in the brown rice. Therefore, the appropriate combined application of Zn and N fertilizers in the field is advantageous for improving both N utilization in plants and Zn nutrition in humans with rice-based diets.

CRediT authorship contribution statement

Chenchen Ji: Conceptualization, Methodology, Data curation, Writing - original draft. **Junli Li:** Methodology, Data curation. **Cuncang Jiang:** Data curation, Writing - review & editing. **Lin Zhang:** Data curation, Writing - review & editing. **Lei Shi:** Funding acquisition, Data curation, Writing - review & editing. **Fangsen Xu:** Data curation, Writing - review & editing. **Hongmei Cai:** Conceptualization, Funding acquisition, Data curation, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Compliance with ethics requirements

This article does not contain any studies with human or animal subjects.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2021.04.005.

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