

# Nitrogen Attenuated Zinc-Mediated Promotion of Rice Tillering Under Low Temperature via Regulating Auxin and Cytokinin Balance

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## Research Article

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# Abstract

## Background and aims

Zinc (Zn) can improve rice resistance to abiotic stress and participate in IAA synthesis. The absorption of Zn is closely related to nitrogen (N) nutrition. However, little is known about the mechanisms by which Zn regulates rice low-temperature resistance and tillering recovery after low-temperature under different N levels.

## Methods

Water culture experiment was conducted with two temperatures (22°C and 12°C), two N levels (1.43 mM and 2.86 mM  $\text{NH}_4\text{NO}_3$ ), and three Zn levels (0.08  $\mu\text{M}$ , 0.15  $\mu\text{M}$  and 0.30  $\mu\text{M}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ).

## Results

Low-temperature decreased rice tillering, which was further exacerbated at high N levels. Increasing Zn application could improve rice low-temperature resistance under normal N levels, enhance nutrient absorption, improve tiller bud cytokinin (CTK) concentration and CTK/IAA ratio, finally accelerate tillering recovery one week before normal Zn treatment. High N attenuated the contribution of Zn under low temperature, but moderate Zn was beneficial to tillering recovery by regulating the balance of tiller bud IAA and CTK concentration, and IAA transport.

## Conclusions

Increasing Zn application improved rice tolerance to low-temperature stress and promoted tillering recovery, which was aggravated under high N levels. However, appropriate Zn application under high N level was conducive to breaking tiller dormancy and promoting tillering growth spurts when recovering to a normal temperature, which was related to the hormone balance and nutrient absorption synergistic regulation by N and Zn.

## Introduction

Rice is the most important food crop, feeding almost 1/2 of the human population (Pittol et al. 2018; Zhang et al. 2017), while rice is sensitive to low temperature (Ma et al. 2015; Reddy et al. 2021). Global climate change has led to frequent occurrence of low temperature damage, which ultimately reduces rice yield (Dar et al. 2021; Pandit et al. 2017; Pradhan et al. 2019). Heilongjiang, one of main rice production regions in China, is a typical high latitude rice growing area (Nie et al. 2019a; Zhang et al. 2015). Low temperature during the vegetative stage is the main factor limiting stable rice production in Heilongjiang

Province (Liu et al. 2019; Shimono et al. 2007). Hence, it is crucial to find approaches to compensate for the reduction of rice production caused by low temperatures.

Low temperature during the vegetative stage affects the carbon and nitrogen (N) metabolism, inhibits root nutrient uptake, reduces rice tiller growth rate and subsequently decreases the number of tillers (Liu et al. 2019; Shimono et al. 2012; Shimono et al. 2002). Meanwhile, low temperature weakens the process of dry matter synthesis and transformation, resulting in a prolonged growth period, and finally reduces the rice yield (Shimono et al. 2007). 15°C low temperature stress decreases rice tillering, especially for the temperature-sensitive rice varieties, and 12°C has a strong depression on tillering for even insensitive varieties (Liu et al. 2019; Reddy et al. 2021). Moreover, the depression effect of low temperature on rice tillering is associated with the duration of the low temperature, that is to say the longer the low temperature lasts, the more harmful it is to rice tiller number and growth rate (Liu et al. 2019; Sperotto et al. 2013). However, some studies have suggested that low temperatures during the vegetative stage delayed tiller emergence and the tillers could begin to grow normally after the temperature increases and quickly recover to normal levels (Liu et al. 2019).

Zinc (Zn), one of the essential micronutrients for plant growth and development, plays a fundamental role in several critical cellular functions, such as protein metabolism, gene expression, structural and functional integrity of the biomembranes, and photosynthetic carbon metabolism (Cakmak 2000; Cakmak et al. 2010a; Marschner 1995). Under abiotic stress conditions, Zn acts as the metal component of copper/zinc-superoxide dismutase (SOD) and can scavenge  $O_2^- \cdot$  to control reactive oxygen species (ROS) production, thus resulting in less damage to cell membranes and enhanced plant stress tolerance (Rehman et al. 2012; Su et al. 2020). The absorption of Zn by plants is a process of thermodynamic passive transmembrane absorption. Low temperatures reduce the membrane electric potential and inhibit the absorption of Zn, and low temperature reduces the accumulation of Zn in many crops (Giordano et al. 1974; Hart 1998; Kochian 1993). Increasing Zn application can alleviate the damage to plants caused by stress, such as salinity and drought, by regulating the osmotic balance, the antioxidant defense system and flavonoid concentrations etc. (Rameshraddy et al. 2017; Su et al. 2020; Tufail et al. 2018). Additionally, as a component of tryptophan synthases, Zn affects the synthesis of auxin (IAA) and is therefore closely related to rice tiller growth and development (Elshayb et al. 2021; Huang et al. 2020; Liu et al. 2011a). When suffering to Zn-deficient condition, the growth of all rice elongated tiller bud is significantly inhibited compared with plants supplied with sufficient Zn, and the outgrowth of the third tiller is inhibited at day 2 after exposure to Zn deficiency (Mu et al. 2021). There was research showed that Zn could alleviate the negative effects of high temperature stress on wheat yield and quality via enhancing N metabolism (Tao et al. 2018). Our previous study showed that an adequate amount of tillers and an improved tiller rate could be obtained via earlier tiller germination facilitated by Zn application after regreening in cold regions (Zhang et al. 2013). However, the effects of Zn applications under low temperatures on rice tiller generation and recovery are still puzzled.

Appropriate increase of nitrogen fertilizer can increase the number of rice tillers (Wang et al. 2016). Meanwhile, N application can enhance Zn absorption, root-to-shoot translocation and distribution,

increase Zn harvest index, and consequently improve the crop yield (Ji et al. 2021; Manzeke et al. 2020; Phattarakul et al. 2012). Under both glasshouse and field conditions, sufficiently high N application is effective in enhancing grain Zn concentration in wheat (Cakmak et al. 2010b; Kutman et al. 2010). This is primarily because N stimulates the activities of transporter proteins involved in xylem loading and enhances the production of nitrogenous compounds facilitating Zn transport in plants (Curie et al. 2008; Erenoglu et al. 2011; Palmer and Guerinot 2009; Suzuki et al. 2008). However, there also researches reported that a high soil N concentration reduced Zn grain concentrations probably due to a secondary “dilution” of this element in the higher biomass (Miner et al. 2018), which was observed especially for soils with low Zn availability (Zhao et al. 2016). Consequently, the level of N supplied to the crop should be taken into account when applying Zn fertilizer (Cakmak and Kutman 2018; Xue et al. 2019). However, it is still confusing as to whether the effects of Zn on rice tiller recovery after low temperatures are also affected by the N concentration.

Zn and N affect the accumulation of the plant hormones IAA and cytokinin (CTK) by regulating IAA and CTK synthesis and transport (Ding et al. 2012; Gao et al. 2019; Zhang et al. 2016). Generally speaking, IAA and CTK concentrations in plants are increased with increasing Zn or N concentrations (Tamaki and Mercier 2007). Increasing Zn supply under adverse conditions can promote IAA synthesis and increase the plant's resistance (Su et al. 2020); however, excessive CTK will inhibit Zn absorption and transport by rice (Gao et al. 2019). IAA and CTK play important roles in regulating tiller germination and growth, and the CTK/IAA ratio affects tiller buds' activation and dormancy. In general, a higher CTK/IAA ratio is beneficial for the germination of tiller buds (Liu et al. 2011b; Shimizu-Sato and Mori 2002). Studies on the relationship between N and rice tillering have demonstrated that an increased but appropriate dosage of N could induce tiller bud germination by affecting the IAA and CTK concentrations of tiller buds and tiller nodes as well as the expression level of *OsPIN*, an IAA transporter (Xu et al. 2015). Consequently, both Zn and N played important roles in the process by which IAA and CTK regulate tillering growth. However, the hormonal regulation mediated by Zn on rice tillering growth under low temperature stress and its relationship with the N application concentration remain to be explored in depth.

In reference to the knowledge gaps described above, a water culture experiment was conducted with different N and Zn concentrations and temperature treatments. The tiller numbers, nutrient absorption, antioxidant enzyme activity, malonaldehyde (MDA) concentration and hormone metabolism were measured to test these three hypotheses: 1) under low temperature stress, Zn can alleviate the inhibition of rice tiller growth caused by low temperatures, but increasing the application of Zn under high N conditions may aggravate the low temperature damage; 2) during recovery to normal temperature, Zn is more beneficial to rice tillering recovery after low temperature exposure under a high N level due to the synergistic effect between N and Zn; 3) the regulatory effect of Zn on rice tillering at low temperature is mainly mediated by affecting the synthesis and transport of endogenous hormones, which is influenced by the N concentration. Testing these hypotheses on how Zn improves plant low temperature resistance will provide theoretical support for increasing rice production.

## Materials And Methods

## **Rice culture and treatment**

To investigate the effects of Zn on rice low temperature tolerance, tiller recovery and their responses to N, a water culture experiment was conducted in 2019. The experiment was a completely random design with three factors: 1) temperature—two levels, 22.0°C and 12°C; 2) N—two levels, 1.43 mM and 2.86 mM  $\text{NH}_4\text{NO}_3$ ; and 3) Zn—three levels, 0.08  $\mu\text{M}$ , 0.15  $\mu\text{M}$  and 0.30  $\mu\text{M}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ . The rice was a chilling-sensitive variety (Wuyoudao-4) provided by the Institute of Crop Cultivation and Tillage, Heilongjiang Academy of Agricultural Sciences. There were 12 treatments and three biological replicates for each treatment.

The rice seedlings were grown in a nursery until 3-leaf stage. 9 uniform rice plants were selected and transplanted into a small hole in a hydroponic device filled with 6 L International Rice Research Institute (IRRI) nutrient solution after rinsing off the soil attached to the rice root. The hydroponic device is an 8 L PVC container (39 × 8 × 19 cm) with a PVC expansion cap floating on the nutrient solution and eight 3.2 cm in diameter holes in each PVC expansion cap. Two N levels, normal (1.43 mM  $\text{NH}_4\text{NO}_3$ ) and double solution N (2.86 mM  $\text{NH}_4\text{NO}_3$ ), and three Zn levels, half (0.08  $\mu\text{M}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ), normal (0.15  $\mu\text{M}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ) and double solution Zn (0.30  $\mu\text{M}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ), were set. At the 5-leaf stage, the rice seedlings were moved into growth chambers with a low temperature (12°C) or a normal temperature (22°C). The growth chamber was set to a 14-hr light/10-hr dark cycle with light provided by fluorescent lamps at 20,000 lx light intensity. After 7 days of temperature treatment, all rice seedlings were transferred outside with the average temperature of 22°C. The nutrient solution was changed once a week and maintained at pH 5.5 with HCl or NaOH. Rice samples were collected after temperature treatment (ATT), 2 weeks (WAR 2) and 4 weeks (WAR 4) after recovery to normal temperature.

## **Rice tillering and plant biomass measurement**

The rice tiller numbers were measured before temperature treatment (BTT), ATT, and weekly after recovery to a normal temperature. After sampling, the samples were rinsed with distilled water and dried with filter paper. The number of tillers was measured then plants were placed in an oven at 105°C for 30min, dried at 85°C for 48h and weighed to determine their dry weights. The tiller growth rate was calculated as the tiller number changes per week.

## **Determination of plant Zn concentration**

The measurement of Zn concentration was performed according to Gao et al. (2019). The dried plant samples were crushed, digested by 0.1M HCl and analyzed using an ICP-OES (PerkinElmer, USA), and measured the Zn concentration of shoot and root separately.

## **Determination of plant total N concentration**

The measurement of total N was performed according to Nelson and Sommers (1973). The dried plant samples were crushed, digested by  $\text{H}_2\text{SO}_4\text{-H}_2\text{O}_2$  and analyzed using an Auto Analyzer 3 (Bran + Luebbe,

Germany). The total amount of N per plant was calculated based on the N concentration and the dry biomass of the shoot.

### **Determination of MDA concentration**

The concentration of MDA was determined according to Xiong et al. (Xiong et al. 2013). Put 0.1 g of fresh leaves in a centrifuge tube, freeze and grind with liquid nitrogen, add 2 mL of phosphate buffer (0.05 M, pH 7.8, a mixture of  $\text{Na}_2\text{HPO}_4$  and  $\text{NaH}_2\text{PO}_4$ ), and centrifuge for 20 minutes at 10,000 rpm at 4°C. Collect the supernatant to be the crude enzyme solution. Take 1 mL of crude enzyme solution in a test tube, add 2 mL of 0.6% TBA. For the control group, 1 mL of deionized water was used instead of the crude enzyme solution. After mixing the sample and reagents, react for 15 minutes in a boiling water bath, quickly cool down and centrifuge, and take the supernatant to measure the absorbance at 600 nm, 532 nm and 450 nm.

### **Antioxidant enzyme activity determination**

Take about 0.1 g of fresh leaf tissue in a centrifuge tube, freeze and grind with liquid nitrogen, add 2 mL of phosphate buffer (0.05 M, pH 7.8, a mixture of  $\text{Na}_2\text{HPO}_4$  and  $\text{NaH}_2\text{PO}_4$ ). The mixture was centrifuged at 10,000 rpm for 20 minutes at 4°C, and the supernatant was collected.

The superoxide dismutase (SOD) activity according to Pan et al. (Pan et al. 2013). Take 20  $\mu\text{L}$  of crude enzyme solution and add 2.95 mL SOD reaction mixture. The SOD reaction mixture contains 0.05 M pH 7.8 phosphate buffer, 130 mM Met, 750  $\mu\text{M}$  NBT, 100  $\mu\text{M}$  EDTA- $\text{Na}_2$ , 20  $\mu\text{M}$  FD and deionized water. For the control group and the blank group, 20  $\mu\text{L}$  of 0.05 M pH 7.8 phosphate buffer was used instead of the crude enzyme solution. The samples were incubated at 30°C and 40% light for 20 minutes. The blank group was protected from light during the incubation. After the incubation, the samples were immediately shaded to stop the reaction, and the absorbance at 560 nm was measured.

The peroxidase (POD) activity according to Rahnema and Ebrahimzadeh (Rahnema and Ebrahimzadeh 2005). Take 20  $\mu\text{L}$  of crude enzyme solution and add 3 mL POD reaction solution (0.1 M, pH 6.0, mixture of  $\text{Na}_2\text{HPO}_4$ ,  $\text{NaH}_2\text{PO}_4$  and guaiacol) into a cuvette and add 30%  $\text{H}_2\text{O}_2$  before testing. For the control group, 20  $\mu\text{L}$  of 0.05 M pH 7.8 phosphate buffer was used instead of the crude enzyme solution. Record the change in absorbance at a wavelength of 470 nm in one minute.

The catalase (CAT) activity according to Ali et al. (Ali et al. 2002). Take 100  $\mu\text{L}$  of crude enzyme solution in a cuvette and add 2.5 mL of CAT reaction solution. The CAT reaction solution contains 0.1 M, pH 7.0, a mixture of  $\text{Na}_2\text{HPO}_4$  and  $\text{NaH}_2\text{PO}_4$  and 0.1 M  $\text{H}_2\text{O}_2$  solution. For the control group, 100  $\mu\text{L}$  of 0.05 M pH 7.8 phosphate buffer was used instead of the crude enzyme solution. Measure and record the one-minute change in absorbance at 240 nm.

### **Measurement of hormone concentration**

The hormone concentration was determined according to Hou et al. (Hou et al. 2008) with some modifies. Sample processing method and improve and optimize. Weigh 1 g of fresh tiller buds, add 5 mL of pre-cooled 80% methanol, grind into a slurry in an ice bath, seal in plastic wrap and cold soak overnight at 4°C. Then the supernatant was obtained by centrifugation at 8,000 rpm/min for 10 minutes at 4°C. The residue was added to 4 mL of 80% cold methanol and centrifuged for 10 minutes, and the supernatant was combined. The whole filtrate was concentrated under reduced pressure at 40°C to 1/3 of the original volume, and 30 mL petroleum ether was added for extraction and decolorization three times, and the ether phase was discarded. The aqueous phase was extracted 3 times with 20 mL ethyl acetate, the ester phases were combined and evaporated to dryness under reduced pressure at 40°C. Add 2 mL of acetic acid solution with pH 3.5, purify through Sep-Pak C18 cartridge, eluting with methanol, collect and concentrate to dryness under reduced pressure at 40°C. Dissolve with mobile phase and dilute to 2 mL, filter through 0.45 µm microporous membrane, and analyze by HPLC.

### **Determination of quantitative gene expression**

Total RNA was extracted from frozen rice plants (approximately 100 mg) using TRIzol reagent and an RNA Purification Kit (Invitrogen, Carlsbad, CA, USA), including DNase treatment, according to the manufacturer's protocol. Total RNA was quantified using a spectrophotometer following electrophoresis on a 0.8% (w/v) agarose gel to assess the concentration and integrity of each sample. Approximately 1 µg of total RNA was transcribed into cDNA using Superscript III Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). The quality of the cDNA was assessed by qRT-PCR using primers for the *Os18S rRNA* genes.

qRT-PCR was performed using an Agilent Mx3000 P Analyzer (Agilent Technologies Ltd., Santa Clara, CA, USA) in a 15 µL reaction volume containing 1 µL cDNA, 2 µL primer mix, and 7.5 µL SYBR Green Master Mix (Agilent Technologies Ltd., Santa Clara, CA, USA). The cycle number was adapted for rice root and AMF. For rice genes, 35 cycles were performed, and AMF genes were amplified with 50 cycles. qRT-PCR was performed on three independent biological samples and three technical replicates. The IAA key gene expression primers were referred to Xu et al. (Xu et al. 2017) and the CTK key gene expression primers were determined according to Ding et al. (Ding et al. 2014), and all primer sequences are shown in Table S1. The comparative  $2^{-\Delta\Delta CT}$  method was used to measure changes in the expression of selected genes relative to untreated controls (Winer et al. 1999).

### **Statistical analysis**

Statistical analysis and correlation analysis were performed using SPSS 25 software. Multiway ANOVA was used to analyze the interaction between temperature, N and Zn, and multiple comparisons were performed by the LSD method. The significance level was 5%, and the LSD (0.05) value was obtained by SAS 8.01. The values in the figures and tables are represented as the means ± standard errors.

## **Results**

## Rice tiller number

There was a significant interaction of T × N × Zn on tiller number at WAR 2-WAR 4 (Table 1). Low temperature significantly suppressed the number of tillers, which were 23.66% ( $P < 0.05$ ) and 42.03% ( $P < 0.05$ ) lower under normal and high N conditions, respectively (Fig. 1). Increasing Zn application increased the rice tiller number by 5.54% under normal N conditions, while rice tiller decreased by 19.43% ( $P < 0.05$ ) under high N conditions.

Table 1  
Results of multiway ANOVA analyses of rice tiller growth, Zn absorption, N absorption, MDA concentration and antioxidase activities.

Parameters	Stage	Factors						
		T	N	Zn	N×Zn	T×N	T×Zn	T×N×Zn
Tiller number	ATT	**	**	ns	**	**	ns	ns
	WAR 1	**	**	ns	**	**	ns	ns
	WAR 2	**	**	ns	**	**	*	*
	WAR 3	**	**	**	ns	ns	**	*
	WAR 4	**	*	**	*	ns	**	*
Tiller growth rate	BTT-ALT	**	ns	ns	**	**	ns	ns
	ALT-WAR 2	**	ns	**	**	ns	**	**
	WAR 2-WAR 4	**	**	**	**	**	**	**
Zn shoot concentration	ATT	**	**	**	**	**	**	*
	WAR 2	**	**	**	ns	ns	ns	ns
	WAR 4	**	**	**	**	**	*	*
Zn root concentration	ATT	**	**	**	ns	**	**	**
	WAR 2	**	**	**	ns	ns	*	**
	WAR 4	**	**	**	ns	*	ns	*
N accumulation	ATT	**	*	**	**	**	**	**
	WAR 2	**	**	**	**	**	ns	**
	WAR 4	**	**	**	**	**	ns	*
N content	ALT	ns	**	ns	**	**	**	**
	WAR 2	**	**	ns	ns	**	ns	*
	WAR 4	**	**	**	ns	*	**	ns
MDA	ATT	**	ns	ns	**	**	**	**
	WAR 2	**	ns	**	ns	ns	ns	ns
	WAR 4	ns	**	**	**	ns	ns	ns

BTT represents before temperature treatment, ATT represents after temperature treatment, WAR represents weeks after recovery to normal temperature. \* and \*\* represent significance at  $P < 0.05$  and  $P < 0.01$ , respectively, and ns represents no significance.

Parameters	Stage	Factors						
		T	N	Zn	N×Zn	T×N	T×Zn	T×N×Zn
SOD	ATT	**	**	**	*	**	**	*
	WAR 2	**	**	**	ns	ns	ns	ns
	WAR 4	**	**	**	ns	**	*	ns
CAT	ATT	**	ns	**	ns	**	**	**
	WAR 2	**	**	**	**	ns	**	ns
	WAR 4	**	**	**	ns	ns	ns	ns
POD	ATT	**	**	ns	**	ns	**	**
	WAR 2	**	**	**	**	ns	ns	**
	WAR 4	ns	**	**	**	ns	ns	ns

BTT represents before temperature treatment, ATT represents after temperature treatment, WAR represents weeks after recovery to normal temperature. \* and \*\* represent significance at  $P < 0.05$  and  $P < 0.01$ , respectively, and ns represents no significance.

At WAR 2, low temperature still had a significant effect on the rice tiller number. Compared with the normal temperature treatment, the number of tillers decreased by 16.29% ( $P < 0.05$ ) under normal N levels and by 38.84% ( $P < 0.05$ ) under high N levels. Under normal N levels, reducing the Zn supply decreased the tiller number by 12.81% ( $P < 0.05$ ), while increasing the Zn level had no significant effect on the tiller number. There was an opposite trend under high N conditions: when increasing the application of Zn, the rice tiller number was still 14.19% ( $P < 0.05$ ) lower than the normal N level.

At WAR 3, low temperature still decreased the rice tiller number by 6.75% ( $P < 0.05$ ) under normal N conditions, and the rice tiller recovered until WAR 4, but with increased Zn application it recovered to the normal level at WAR 3. Under high N levels, the rice tiller number recovered to normal levels at WAR 3 even without increasing the Zn supply. However, the rice tiller number at WAR 4 was still significantly lower than the normal level if the Zn application was reduced at both N levels.

The dry matter accumulation in the shoot and root of rice under different temperature, N and Zn treatments had similar trends with the rice tiller number during these three periods (Fig. S1). At WAR4, shoot dry matter could recover to normal level under normal N conditions, which was still significantly lower than normal temperature for high N treatments. Increasing Zn application could help the accumulation of rice dry matter, while rice shoot and root dry matter weight significantly decreased when reducing the supply of Zn. Tiller growth rate

There were significant interactions of T × N × Zn on the tiller growth rate at ATT-WAR 2 and WAR 2-WAR 4 (Table 1). Low temperature significantly decreased the rice tiller growth rate. Under the normal N level, increasing the Zn concentration was beneficial and increased the rice tiller growth rate; however, the rice

tiller growth rate was significantly decreased with an increase of the Zn concentration under a high N level (Fig. 2).

During ATT-WAR 2, under normal N levels, increasing the Zn application increased the rice tiller growth rate, while the rice tiller growth rate decreased by 29.85% ( $P < 0.05$ ) if the Zn concentration was decreased. Increasing the N supply reduced the rice tiller growth rate after the low temperature treatment, and increasing the Zn application further inhibited the rice tiller growth rate, with the rice tiller growth rate decreasing by 44.44% ( $P < 0.05$ ), while decreasing the Zn supply increased the rice tiller growth rate by 14.29%.

During WAR 2-WAR 4, the rice tiller growth rate after the low temperature treatments was higher than that after the normal temperature treatments, and the rice tiller growth rate increased by 39.34% ( $P < 0.05$ ) and 135.71% ( $P < 0.05$ ) for the normal and high N levels, respectively. Increasing the Zn supply level increased the tiller growth rate by 29.41% ( $P < 0.05$ ) and 10.91% ( $P < 0.05$ ) under normal and high N levels, respectively.

### Shoot and root Zn concentration

T × N × Zn had significant interaction effects on the shoot zinc concentration at ATT and WAR 4, and on the root Zn concentration at all stages (Table 1). Low temperature stress increased rice shoot and root Zn concentration by 13.12% ( $P < 0.05$ ) and 34.59% ( $P < 0.05$ ), respectively at normal N level; and by 62.83% ( $P < 0.05$ ) and 70.14% ( $P < 0.05$ ), respectively at high N level (Fig. 3). Under low temperature stress, increasing Zn application had little effect on rice shoot Zn concentration at normal N condition, but significantly elevated root and shoot Zn concentrations at high N level. At WAR 2 and WAR 4, the shoot and root Zn concentrations of rice were lower than ATT, and high N treatments showed higher Zn concentration than that at normal N level. Additionally, increasing Zn application significantly increased shoot and root Zn concentration both at normal and high N conditions.

In order to identify the relationship between shoot Zn concentration and rice tiller numbers at different temperature and N conditions, the correlation of Zn concentration and tiller was analyzed. At normal N level, there was significant positive correlations between shoot Zn concentration and tiller number both under normal and low temperature conditions (Fig. 4). At high N level, significant negative correlation at ATT and positive correlation at WAR2 and WAR4 was observed under normal temperature conditions. However, there was negative correlations between rice tillers and shoot Zn concentration at ATT and WAR2 under low temperature conditions. And quadratic curve relationship was observed at WAR4, the Zn concentration with the highest rice tiller number was 32.62 mg kg<sup>-1</sup> (Fig. 4f). N absorption

T × N × Zn had a significant interactive effect on N concentration at ATT and WAR 2 (Table 1). Increasing the N levels significantly increased the shoot N uptake not only under normal but also under low temperature conditions (Fig. 5a). The N concentration of rice treated with high N levels decreased by 8.26% ( $P < 0.05$ ) under low temperature treatment and increased by 7.63% ( $P < 0.05$ ) under normal N levels. Under low temperature, increased Zn application increased N uptake by 4.39% ( $P < 0.05$ ) at the

normal N level but decreased N uptake by 5.94% ( $P < 0.05$ ) at the high N level. At WAR 2 and WAR 4, there was little effect of N and Zn on the rice shoot N concentration.

T × N × Zn all had significant interactive effects on N accumulation in all stages (Table 1). Low temperature significantly decreased the rice N accumulation, but there was no significant difference between N levels. Under low temperature conditions, increasing the application of Zn increased the N accumulation by 16.61% ( $P < 0.05$ ) at normal N levels. However, increasing the Zn supply under a high N level decreased the N accumulation by 20.90% ( $P < 0.05$ ), which increased by 20.55% ( $P < 0.05$ ) with decreased Zn application (Fig. 5b).

A negative effect of low temperature on N accumulation remained at WAR 2, which was more severe under high N levels. N accumulation under high N levels recovered to 50.04% ( $P < 0.05$ ) of normal conditions at WAR 2, while N accumulation under normal N levels recovered to 75.04% ( $P < 0.05$ ). Reducing the Zn supply reduced the N accumulation by 39.18% ( $P < 0.05$ ) at normal N levels, which could recover to 89.64% ( $P < 0.05$ ) of the normal levels when increasing the Zn application. Under high N levels, increased Zn application reduced the N accumulation by 15.84% ( $P < 0.05$ ) and 42.11% ( $P < 0.05$ ) under normal conditions.

At WAR 4, the low temperature treatment still significantly inhibited N accumulation under high N levels, while N accumulation under normal N levels had recovered to normal levels. Increasing the Zn supply increased the N accumulation by 24.20% ( $P < 0.05$ ) at normal N levels, but Zn had no significant effect on N accumulation under high N levels. Decreasing the Zn application significantly inhibited the recovery of N accumulation. The correlation analysis showed that there was a significant positive correlation between N accumulation and tiller increment during ATT-WAR 2 and WAR 2-WAR 4 (Fig. 6), and there was also a significant positive correlation between the Zn application and the N accumulation increment under normal N levels (Fig. 7). However, there was a significant positive correlation between the Zn application level and the N accumulation increment only during WAR 2-WAR 4 at high N level (Fig. 7).

### **MDA concentration and antioxidase activities**

There was a significant interactive effect of T × N × Zn on MDA concentration and SOD, CAT and POD activities during the ATT period and on POD activity at WAR 2 (Table 1). Low temperature caused significant MDA accumulation, with increases of 19.00% ( $P < 0.05$ ) and 47.32% ( $P < 0.05$ ) at normal and high N levels, respectively (Table 2). An increased Zn supply reduced the rice MDA concentration by 21.29% ( $P < 0.05$ ) at the normal N level, while it increased the MDA concentration by 21.82% ( $P < 0.05$ ) at the high N level. Under low temperature stress, the activities of POD and CAT in rice leaves at the normal N level increased by 11.44% ( $P < 0.05$ ) and 19.43% ( $P < 0.05$ ), respectively. SOD activities at high N levels increased by 23.32% ( $P < 0.05$ ). Increasing Zn supply enhanced the SOD, POD and CAT activities at normal N levels, which only increased SOD activity by 10.42% ( $P < 0.05$ ) at high N levels while inhibiting the other enzyme activities.

Table 2

MDA concentration and antioxidase activities in rice under different temperature, N and Zn application levels.

Stage	Temperature	Treatment	MDA concentration ( $\mu\text{mol g}^{-1}$ FW)	SOD activity ( $\text{U g}^{-1}$ FW)	CAT activity ( $\Delta 240 \text{ min}^{-1}$ $\text{g}^{-1}$ FW)	POD activity ( $\text{U min}^{-1} \text{g}^{-1}$ FW)		
ATT	22°C	N1Zn1	4.4 ± 0.1	1006 ± 62	48 ± 1	340 ± 12		
		N1Zn2	3.8 ± 0.3	1149 ± 39	52 ± 1	347 ± 3		
		N1Zn3	3.1 ± 0.2	1259 ± 49	52 ± 1	352 ± 11		
		N2Zn1	3.2 ± 0.0	985 ± 20	57 ± 1	372 ± 6		
		N2Zn2	3.4 ± 0.1	1012 ± 11	58 ± 1	378 ± 2		
		N2Zn3	3.6 ± 0.0	1068 ± 10	53 ± 3	358 ± 9		
		N1Zn1	5.1 ± 0.3	1142 ± 33	59 ± 4	341 ± 12		
		N1Zn2	4.5 ± 0.1	1218 ± 33	62 ± 2	387 ± 18		
		N1Zn3	3.6 ± 0.3	1368 ± 24	83 ± 2	445 ± 16		
	12°C	N2Zn1	3.7 ± 0.1	1151 ± 11	66 ± 1	440 ± 13		
		N2Zn2	5.0 ± 0.1	1248 ± 26	61 ± 1	397 ± 0		
		N2Zn3	6.0 ± 0.1	1378 ± 15	56 ± 2	368 ± 9		
		LSD (0.05)		0.5	91	5	29	
		WAR 2	22°C	N1Zn1	3.8 ± 0.6	697 ± 19	51 ± 1	164 ± 19

N1 and N2 represent normal (1.43 mM  $\text{NH}_4\text{NO}_3$ ) and double solution N (2.86 mM  $\text{NH}_4\text{NO}_3$ ) level, respectively; Zn1, Zn2 and Zn3 represent half (0.08  $\mu\text{M}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ), normal (0.15  $\mu\text{M}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ) and double solution Zn (0.30  $\mu\text{M}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ) level, respectively. 22°C represents the normal temperature, and 12°C represents the low temperature. ATT represents after temperature treatment, and WAR represents weeks after recovery to normal temperature. Values are the means ± standard errors for three biological replicates. LSD (0.05) is the least significant difference between treatments at  $P < 0.05$ .

Stage	Temperature	Treatment	MDA concentration ( $\mu\text{mol g}^{-1}$ FW)	SOD activity ( $\text{U g}^{-1}$ FW)	CAT activity ( $\Delta 240 \text{ min}^{-1} \text{ g}^{-1}$ FW)	POD activity ( $\text{U min}^{-1} \text{ g}^{-1}$ FW)
		N1Zn2	$3.5 \pm 0.1$	$770 \pm 21$	$51 \pm 1$	$175 \pm 23$
		N1Zn3	$3.1 \pm 0.1$	$887 \pm 49$	$59 \pm 2$	$252 \pm 18$
		N2Zn1	$3.5 \pm 0.0$	$583 \pm 7$	$52 \pm 1$	$180 \pm 9$
		N2Zn2	$3.4 \pm 0.0$	$693 \pm 8$	$51 \pm 1$	$215 \pm 4$
		N2Zn3	$2.8 \pm 0.0$	$793 \pm 5$	$48 \pm 0$	$288 \pm 0$
	12°C	N1Zn1	$4.2 \pm 0.1$	$896 \pm 69$	$51 \pm 3$	$309 \pm 18$
		N1Zn2	$3.6 \pm 0.1$	$954 \pm 22$	$56 \pm 2$	$314 \pm 9$
		N1Zn3	$3.4 \pm 0.2$	$1050 \pm 60$	$63 \pm 4$	$344 \pm 13$
		N2Zn1	$3.9 \pm 0.1$	$786 \pm 13$	$50 \pm 1$	$296 \pm 5$
		N2Zn2	$3.7 \pm 0.1$	$897 \pm 23$	$52 \pm 1$	$384 \pm 4$
		N2Zn3	$3.4 \pm 0.0$	$990 \pm 9$	$56 \pm 2$	$442 \pm 12$
LSD (0.05)			0.6	94	6	40
WAR 4	22°C	N1Zn1	$3.1 \pm 0.3$	$465 \pm 62$	$31 \pm 4$	$257 \pm 10$
		N1Zn2	$2.5 \pm 0.3$	$645 \pm 38$	$39 \pm 4$	$328 \pm 13$
		N1Zn3	$2.3 \pm 0.0$	$657 \pm 56$	$43 \pm 1$	$372 \pm 18$
		N2Zn1	$3.3 \pm 0.0$	$871 \pm 18$	$45 \pm 1$	$336 \pm 25$
		N2Zn2	$3.2 \pm 0.1$	$972 \pm 10$	$47 \pm 1$	$395 \pm 30$
		N2Zn3	$2.9 \pm 0.1$	$1007 \pm 14$	$49 \pm 3$	$391 \pm 37$
	12°C	N1Zn1	$3.1 \pm 0.1$	$909 \pm 50$	$39 \pm 1$	$275 \pm 28$
		N1Zn2	$2.7 \pm 0.0$	$987 \pm 33$	$44 \pm 2$	$302 \pm 26$

N1 and N2 represent normal (1.43 mM  $\text{NH}_4\text{NO}_3$ ) and double solution N (2.86 mM  $\text{NH}_4\text{NO}_3$ ) level, respectively; Zn1, Zn2 and Zn3 represent half (0.08  $\mu\text{M}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ), normal (0.15  $\mu\text{M}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ) and double solution Zn (0.30  $\mu\text{M}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ) level, respectively. 22°C represents the normal temperature, and 12°C represents the low temperature. ATT represents after temperature treatment, and WAR represents weeks after recovery to normal temperature. Values are the means  $\pm$  standard errors for three biological replicates. LSD (0.05) is the least significant difference between treatments at  $P < 0.05$ .

Stage	Temperature	Treatment	MDA concentration ( $\mu\text{mol g}^{-1}$ FW)	SOD activity ( $\text{U g}^{-1}$ FW)	CAT activity ( $\Delta 240 \text{ min}^{-1} \text{ g}^{-1}$ FW)	POD activity ( $\text{U min}^{-1} \text{ g}^{-1}$ FW)
		N1Zn3	$2.3 \pm 0.3$	$1056 \pm 30$	$44 \pm 5$	$374 \pm 8$
		N2Zn1	$3.4 \pm 0.1$	$991 \pm 9$	$51 \pm 1$	$336 \pm 3$
		N2Zn2	$3.3 \pm 0.0$	$1018 \pm 15$	$51 \pm 1$	$344 \pm 14$
		N2Zn3	$2.9 \pm 0.1$	$1094 \pm 14$	$53 \pm 1$	$372 \pm 13$
		LSD (0.05)	0.5	96	7	63
<p>N1 and N2 represent normal (1.43 mM <math>\text{NH}_4\text{NO}_3</math>) and double solution N (2.86 mM <math>\text{NH}_4\text{NO}_3</math>) level, respectively; Zn1, Zn2 and Zn3 represent half (0.08 <math>\mu\text{M ZnSO}_4 \cdot 7\text{H}_2\text{O}</math>), normal (0.15 <math>\mu\text{M ZnSO}_4 \cdot 7\text{H}_2\text{O}</math>) and double solution Zn (0.30 <math>\mu\text{M ZnSO}_4 \cdot 7\text{H}_2\text{O}</math>) level, respectively. 22°C represents the normal temperature, and 12°C represents the low temperature. ATT represents after temperature treatment, and WAR represents weeks after recovery to normal temperature. Values are the means <math>\pm</math> standard errors for three biological replicates. LSD (0.05) is the least significant difference between treatments at <math>P &lt; 0.05</math>.</p>						

During the WAR 2 stage, the MDA concentration of the low temperature treatment had recovered to normal levels, and the SOD and POD activities were significantly higher than those of the normal temperature treatment at both N levels. Regardless of the normal or high N level, increasing the Zn supply could improve the SOD, POD and CAT activities, but N had no significant effect on MDA accumulation. At WAR 2 and WAR 4, an increased Zn supply was beneficial in reducing the MDA concentration at both normal and high N levels, but there was no significant difference. At WAR 4, there was little difference in enzyme activities among the treatments.

### Tiller bud IAA and CTK concentration

There was an interaction of T × N × Zn on tiller bud IAA concentration (Table 4). Low temperature treatment increased the tiller bud IAA concentration under normal N levels by 29.63% ( $P < 0.05$ ) compared with normal temperature treatment, while CTK/IAA ratio decreased by 17.42% ( $P < 0.05$ ); the IAA and CTK concentrations under high N levels increased by 105.52% ( $P < 0.05$ ) and 94.05% ( $P < 0.05$ ), respectively, but CTK/IAA ratio was not significantly different from that under normal temperature conditions (Table 3). Although increasing the Zn supply had no significant effect on the tiller bud CTK and IAA concentrations at normal N levels, the CTK/IAA ratio increased by 7.03%. Increasing the Zn application at a high N level increased the IAA concentration by 30.54% ( $P < 0.05$ ) but had no effect on the CTK concentration, and the CTK/IAA ratio decreased by 27.13% ( $P < 0.05$ ).

Table 3  
The concentrations of IAA, CTK and CTK/IAA ratio in tiller buds.

Stage	Temperature	Treatment	IAA (ng g <sup>-1</sup> FW)	CTK (ng g <sup>-1</sup> FW)	CTK/IAA ratio	
ATT	22°C	N1Zn1	87 ± 3	134 ± 4	1.5 ± 0.1	
		N1Zn2	279 ± 4	116 ± 9	1.6 ± 0.0	
		N1Zn3	90 ± 6	162 ± 12	1.8 ± 0.1	
	12°C	N2Zn1	N2Zn1	95 ± 3	122 ± 4	1.4 ± 0.1
			N2Zn2	90 ± 3	120 ± 4	1.3 ± 0.1
			N2Zn3	126 ± 2	162 ± 6	1.2 ± 0.0
		N1Zn1	N1Zn1	89 ± 5	119 ± 16	1.1 ± 0.0
			N1Zn2	102 ± 5	130 ± 2	1.3 ± 0.1
			N1Zn3	107 ± 4	146 ± 17	1.4 ± 0.1
N2Zn1	N2Zn1	113 ± 3	152 ± 4	1.4 ± 0.1		
	N2Zn2	185 ± 5	232 ± 6	1.3 ± 0.7		
	N2Zn3	242 ± 6	227 ± 7	0.9 ± 0.1		
LSD (0.05)			12	27	0.2	
WAR 2	22°C	N1Zn1	208 ± 12	232 ± 16	1.1 ± 0.1	
		N1Zn2	200 ± 5	165 ± 10	0.8 ± 0.1	
		N1Zn3	194 ± 10	210 ± 15	1.1 ± 0.1	
		N2Zn1	183 ± 8	113 ± 11	0.6 ± 0.0	
		N2Zn2	213 ± 9	129 ± 4	0.6 ± 0.1	
		N2Zn3	223 ± 5	146 ± 6	0.7 ± 0.0	
	12°C	N1Zn1	179 ± 15	165 ± 14	0.9 ± 0.1	
		N1Zn2	132 ± 15	151 ± 4	1.2 ± 0.2	

N1 and N2 represent normal (1.43 mM NH<sub>4</sub>NO<sub>3</sub>) and double solution N (2.86 mM NH<sub>4</sub>NO<sub>3</sub>) level, respectively; Zn1, Zn2 and Zn3 represent half (0.08 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O), normal (0.15 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O) and double solution Zn (0.30 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O) level, respectively. 22°C represents the normal temperature, and 12°C represents the low temperature. ATT represents after low temperature, and WAR represents the weeks after recovery to normal temperature. Values are the means ± standard errors for three biological replicates. LSD (0.05) is the least significant difference between treatments at *P* < 0.05.

Stage	Temperature	Treatment	IAA (ng g <sup>-1</sup> FW)	CTK (ng g <sup>-1</sup> FW)	CTK/IAA ratio
		N1Zn3	126 ± 15	130 ± 5	1.1 ± 0.1
		N2Zn1	213 ± 8	134 ± 6	0.6 ± 0.0
		N2Zn2	133 ± 9	116 ± 1	0.9 ± 0.1
		N2Zn3	101 ± 8	135 ± 1	1.4 ± 0.1
LSD (0.05)			30	28	0.3
WAR 4	22°C	N1Zn1	213 ± 11	143 ± 8	0.7 ± 0.1
		N1Zn2	175 ± 17	144 ± 8	0.8 ± 0.0
		N1Zn3	151 ± 15	155 ± 16	1.0 ± 0.0
		N2Zn1	166 ± 7	137 ± 5	0.9 ± 0.0
		N2Zn2	160 ± 3	144 ± 1	0.9 ± 0.0
		N2Zn3	127 ± 6	143 ± 0	1.1 ± 0.1
	12°C	N1Zn1	106 ± 5	150 ± 7	1.4 ± 0.0
		N1Zn2	127 ± 25	149 ± 3	1.3 ± 0.2
		N1Zn3	79 ± 1	149 ± 4	1.9 ± 0.1
		N2Zn1	134 ± 5	142 ± 9	1.1 ± 0.0
		N2Zn2	144 ± 2	145 ± 8	1.0 ± 0.1
		N2Zn3	186 ± 1	150 ± 8	0.8 ± 0.1
LSD (0.05)			32	23	0.2

N1 and N2 represent normal (1.43 mM NH<sub>4</sub>NO<sub>3</sub>) and double solution N (2.86 mM NH<sub>4</sub>NO<sub>3</sub>) level, respectively; Zn1, Zn2 and Zn3 represent half (0.08 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O), normal (0.15 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O) and double solution Zn (0.30 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O) level, respectively. 22°C represents the normal temperature, and 12°C represents the low temperature. ATT represents after low temperature, and WAR represents the weeks after recovery to normal temperature. Values are the means ± standard errors for three biological replicates. LSD (0.05) is the least significant difference between treatments at *P* < 0.05.

Table 4

Results of multiway ANOVA for analyses of differences in the content of IAA, CTK, CTK/IAA ratio and the expression of key genes involved in hormone metabolism.

Parameters	Stage	Factors						
		T	N	Zn	N×Zn	T×N	T×Zn	T×N×Zn
IAA	ATT	**	**	**	**	**	**	**
	WAR 2	**	ns	**	ns	ns	**	**
	WAR 4	**	ns	*	**	**	**	*
CTK	ATT	**	**	**	**	**	**	ns
	WAR 2	**	**	*	**	**	ns	**
	WAR 4	ns	ns	ns	ns	ns	ns	ns
CTK/IAA ratio	ATT	**	**	ns	**	**	*	ns
	WAR 2	**	**	**	*	*	**	*
	WAR 4	**	**	**	**	**	ns	**
<i>OsPIN1b</i>	ATT	**	**	**	**	**	**	**
	WAR 2	**	ns	**	**	**	**	ns
<i>OsYUCCA1</i>	ATT	**	ns	**	**	**	**	**
	WAR 2	ns	**	**	**	ns	ns	**
<i>OsYUCCA2</i>	ATT	**	*	**	**	ns	ns	**
	WAR 2	**	**	**	ns	**	**	**
<i>OsYUCCA4</i>	ATT	**	**	**	**	*	**	**
	WAR 2	**	**	*	**	**	ns	**
<i>OsIPT1</i>	ATT	**	**	**	**	**	**	**
	WAR 2	**	ns	**	**	**	**	**
<i>OsIPT2</i>	ATT	**	**	**	**	ns	**	ns
	WAR 2	**	ns	**	**	ns	**	*

ATT represents after temperature treatment, and WAR represents weeks after recovery to normal temperature. \* and \*\* present significance at  $P < 0.05$  and  $P < 0.01$ , respectively, and ns means no significance.

There was an interaction of T × N × Zn on tiller bud IAA and CTK concentration and CTK/IAA ratio at WAR 2, and IAA concentration and CTK/IAA ratio at WAR 4 (Table 4). At WAR 2, the tiller bud IAA concentration after the low temperature treatment decreased significantly, but there was little change in the CTK

concentration (Table 3). The effect of Zn on the CTK concentration of the low temperature treatment was not significant; reducing the Zn supply significantly increased the tiller bud IAA concentration at normal and high N levels, but increasing the Zn supply under high N level decreased the IAA concentration by 23.74% ( $P < 0.05$ ) and increased CTK/IAA ratio by 53.41% ( $P < 0.05$ ). However, Zn had little effect on CTK/IAA ratio under normal N conditions. N and Zn had no significant effect on the CTK ratio concentration at WAR 4. Increasing the Zn supply decreased the IAA concentration by 38.16% ( $P < 0.05$ ) and increased CTK/IAA ratio by 27.59% ( $P < 0.05$ ) at a normal N level; increasing the Zn supply at a high N level significantly increased the IAA concentration by 29.14% ( $P < 0.05$ ), which had no significant effect on CTK/IAA ratio.

### Genes expression related to hormone metabolism

T × N × Zn had an interaction effect on the expression levels of the key IAA and CTK metabolism genes, except *OsIPT2*. At WAR 2, T × N × Zn had an interaction effect on *OsIPT2* expression (Table 4). Low temperature inhibited the expression of *OsYUCCA1* but triggered the expression of *OsYUCCA2*, *OsYUCCA4*, *OsIPT1* and *OsIPT2* (Fig. 8a). Increased Zn application significantly promoted the expression levels of *OsPIN1b*, *OsYUCCA2* and *OsYUCCA4* under normal N conditions, but the expression levels of the three genes were significantly reduced under high N concentrations. In addition, increasing the Zn supply significantly inhibited the expression of *OsYUCCA1* at both N levels and promoted the expression of *OsIPT1* and *OsIPT2*. At WAR 2, increased Zn application significantly promoted the expression of *OsYUCCA2*, *OsPIN1b*, and *OsIPT2* at normal N levels but decreased the expression of *OsYUCCA1*, *OsYUCCA4*, and *OsIPT1* (Fig. 8b). Increasing the Zn concentration inhibited the expression of *OsYUCCA1*, *OsYUCCA4*, and *OsIPT1* at high N levels but promoted the expression of *OsPIN1b*, *OsYUCCA2* and *OsIPT2*.

## Discussion

Rice tillering is an important guarantee of rice quality and high yield (Peng et al. 2015; Wang et al. 2018). Low temperature exposure inhibits rice tiller generation, reduces the tiller number, and then decreases rice yield (Liu et al. 2019). Zn is an essential element in rice tillering development and it can enhance plant resistance to abiotic stress, its uptake and utilization by rice is closely related to N status in the environment (Nie et al. 2019b; Su et al. 2020). However, the effect of Zn on low temperature resistance and tiller recovery is still unclear, especially the effect of the N supply concentration on the process by which Zn affects tiller growth and recovery.

Zn improved rice low temperature resistance and accelerated tillering recovery at normal N level

Studies have shown that exposure to a low temperature (15°C) during the early stage of growth could damage rice plants (Reddy et al. 2021), exposure to 12°C could reduce seedling biomass, inhibit nutrient uptake, and decrease the number of tillers (Liu et al. 2019). In addition to the above physical damage, low temperature stress also induced the generation of large amounts of reactive oxygen species (ROS), increased MDA accumulation and disturbed carbon and N metabolism (Han et al. 2017; Liu et al. 2019).

The results of this experiment also demonstrated that 7 days exposure to a low temperature (12°C) significantly increased the MDA concentration, declined root activities (data not shown), decreased the N absorption (Fig. 5), inhibited tillering growth (Fig. 2), and finally decreased the number of rice tillers number (Figs. 1 and 9) and dry matter accumulation (Fig. S1). Meanwhile, the antioxidant enzyme activity (SOD, POD, CAT) was significantly increased under low temperature stress (Table 2).

As important catalytic factors and essential components of many enzymes and proteins, Zn is an essential micronutrient for rice growth and development (Rehman et al. 2012). At the same time, Zn can also improve the resistance of plants to abiotic stress by scavenging reactive oxygen species. Studies have shown that Zn plays important roles under drought, heavy metal, salinity, and UV stresses (Huang et al. 2018; Su et al. 2020; Tufail et al. 2018). However, the effect of Zn on rice resistance to low temperature is still unclear. The results of this study showed that the activity of SOD and POD in rice increased significantly and that the MDA concentration significantly decreased after increasing Zn supply under low temperature stress (Table 2), indicating that Zn could significantly improve rice low temperature tolerance and promote N absorption. The same conclusion was obtained in a previous study of heavy metal stress (Upadhyay and Panda 2010).

Low temperature during the vegetative stage significantly depresses rice tillering (Liu et al. 2019). Although short duration or low strength low temperature exposure had little effect on the rice tiller number, a long duration or strong strength low exposure temperature irreversible damage on rice tillering, and the rice tiller number could not recover to normal level even for low temperature insensitive cultivars (Liu et al. 2019). Consequently, it is important to improve rice low temperature resistance and find appropriate approaches to reduce low temperature damage to rice tillers and promote the recovery of tillers after exposure to low temperature. In this study, low temperature reduced rice tiller numbers by 23.66% but increasing Zn application increased the tiller number by 5.54% (Fig. 1). Rice tiller numbers recovered to normal levels at WAR 4 without increasing Zn supply but could recover one week earlier if the Zn application was increased. It also can be seen from the tiller growth rate that Zn supply significantly increased the tiller growth rate and promoted tiller growth (Fig. 2), demonstrating that Zn could increase the number of rice tillers after exposure to low temperatures and promote tiller growth and recovery.

Zn enhanced rice tiller recovery by improving nutrient uptake and hormones balance

Tillering growth is inseparable from the supply of N and Zn nutrients (Wang et al. 2016), and our previous study identified that the increase of tiller numbers after exposure to low temperature was significantly and positively correlated with the N accumulation increment (Liu et al. 2019). In cold regions, applying Zn after regreening can also facilitate earlier tiller germination and increase effective panicles number (Zhang et al. 2013). In the current research, increasing Zn application could improve Zn and N absorption at normal N condition, when suffering low temperature stress (Figs. 3 and 5). Significant positive correlation between tiller number and shoot Zn concentration were observed not only under normal temperature but low temperature stress (Fig. 4), and there was a significant positive correlation between N accumulation and tiller number increment after increasing Zn supply at ATT-WAR 2 and WAR 2-WAR 4

(Fig. 6). Meanwhile, there was a significant positive correlation between the Zn application and the N accumulation increment in both periods as well (Fig. 7a and b), indicating that maintaining sufficient Zn and N nutrition was crucial important for rice tillering and promoting nutrient absorption might be one of the possible mechanisms of Zn improving rice tiller growth and recovery after low temperature stress (Fig. 9).

Rice tiller growth mainly consists of two processes: tiller bud germination and elongation (Li et al. 2003; Shao et al. 2019), in which the plant hormones IAA and CTK play important regulatory roles in addition to nutrient requirements (Wang et al. 2019). CTK facilitates tiller development, while IAA is the main hormone regulating apical dominance, which is not conducive to tiller germination at higher concentrations (Choi et al. 2012; Cui et al. 2010; Lv et al. 2018). However, some studies have suggested that tiller bud germination is always accompanied by strengthening of IAA outward transport capacity (Prusinkiewicz et al. 2009). Consequently, the output of IAA in the tiller buds is necessary for tillering germination and growth. In general, CTK/IAA ratio is an important characteristic of axillary bud growth, and a higher CTK/IAA ratio is more favorable for tiller bud generation and elongation (Cui et al. 2010; Wang et al. 2006). Zn participates in the synthesis of IAA from tryptophan, Zn deficiency decreases Zn transporting into tiller bud, negatively affect the synthesis of IAA, which in turn will suppress tiller growth (Mu et al. 2021; Sadeghzadeh 2013; Takaki and Kushizaki 1970). Moreover, Zn could eliminate ROS, so Zn can also inhibit the decomposition of IAA by ROS under stress conditions (Musacchi et al. 2002; Upadhyay and Panda 2010). In this experiment, the IAA and CTK concentrations and CTK/IAA ratio of rice tiller buds decreased significantly with a reduced Zn application (Table 3), and increasing Zn supply increased rice Zn concentration (Fig. 3), promoted the expression of key genes for IAA and CTK synthesis and IAA transport in rice (Figs. 8 and 9), therefore rice still had a higher CTK/IAA ratio under low temperature stress (Table 3). After recovery to normal temperature, increasing Zn application reduced the IAA concentration in the tiller buds, but had less effect on CTK, so a higher CTK/IAA ratio was still presented in the tiller buds (Table 3). This was mainly due to the promotion of *OsPIN1b* expression under increasing Zn application conditions (Fig. 8b), indicating that during the recovery of rice from low temperature stress, Zn promoted IAA transport to other parts, reduced IAA accumulation in tiller buds, increased CTK/IAA ratio, broke the dormancy of the tiller buds, and promoted tiller growth (Fig. 9).

Nitrogen affected the contribution of Zn on rice tiller growth under low temperature

Zn uptake and utilization by plants are both affected by the N application level (Nie et al. 2019b). N could promote the synthesis of nitrogenous compounds that are beneficial for Zn transport and improve Zn uptake by the plant root system (Erenoglu et al. 2011; Ji et al. 2021). Our experiment obtained a similar result that increasing the N supply could significantly increase the Zn concentration of rice (Fig. 3), and the contribution of Zn to rice resistance to low temperature exposure was also affected by the concentration of N. Compared with normal N levels, high N application increased MDA concentrations under low temperature stress (Table 2), reduced the rice tiller growth rate (Fig. 2), and significantly reduced the tiller numbers (Fig. 1). Increasing Zn application caused higher MDA accumulation (Table 2), lower dry matter production (Fig. S1), significantly reduced the tiller growth rate and tiller numbers at high

N level (Fig. 1 and 2). Although increasing Zn application increased shoot and root Zn concentration under high N condition, the increase of Zn concentration might be due to the decrease of dry matter production, and the results of the correlation analysis showed that there was a significant negative correlation between the shoot Zn concentration and the number of tillers when suffering low temperature stress (Fig. 4d). These results suggested that increasing Zn supply at high N level might aggravate the damage of low temperature. Consequently, the ability of Zn help rice resisting to low temperatures was associated with the level of N application, but the internal mechanism is still unclear and needs further study.

The results of plant hormone showed that the IAA concentration of tiller buds treated with high N concentration increased significantly under low temperature stress, an increase in Zn application significantly increased the IAA concentration and then significantly decreased the CTK/IAA ratio (Table 3). The gene expression data also showed that an increased Zn application under high N level suppressed rice *OsPIN1b* expression and reduced the transport of IAA from tiller bud to other parts (Fig. 8a), thus excessive Zn application under high N level led to a higher CTK/IAA ratio. Possible reasons for the above results were that significant higher Zn concentration under high N condition lead to excessive IAA accumulation in tiller bud (Fig. 3 and Table 3). When recovered to normal temperature for 2 weeks, rice shoot and root Zn concentrations were lower than ATT, while rice plants of increasing Zn application under high N condition still had higher Zn concentration, which was negatively affected tiller number (Fig. 4e). Furthermore, increasing Zn application promoted the expression of the key genes for CTK synthesis and IAA transport, resulting in significant decrease in tiller bud IAA concentration and significant increase in CTK/IAA ratio (Table 3 and Fig. 8b). Previous studies have shown that excessively high IAA or low CTK/IAA ratio could cause tiller buds to enter dormancy (Haver et al. 2002). According to the Zn concentration, hormonal changes and tiller recovery, although the tiller number decreased significantly under low temperature stress with the Zn application increasing under high N level, the low temperature did not cause tiller bud death and instead they entered dormancy due to the inhibition of tiller bud germination and elongation caused by high IAA concentration. After the low temperature stress was relieved, the shoot Zn concentration and IAA concentration in the tiller buds were reduced, and CTK/IAA ratio increased along with the enhancement of IAA transport. Thus, the dormancy of the tiller buds was broke, tillering growth was spurred at WAR 3 and then quickly recovered to normal levels. However, there was no significant difference of tiller numbers between normal and high Zn levels (Fig. 1). Consequently, the rice tiller number could recover to normal level with a moderate amount of Zn application at high N level. Nevertheless, this result was only verified from the hormonal perspective, and the internal mechanisms need to be further explored from morphological and molecular perspectives.

Although high N levels attenuated the contribution of Zn in improving rice low temperature resistance, rice growth after recovery to normal temperature still required an additional nutrient supply (Liu et al. 2019). An increased N supply increases the chlorophyll concentration, enhances the rice photosynthetic rate and accelerates carbon and N metabolism, and then enhances the leaf area index, biomass, and tiller number, therefore alleviating the low temperature damage that occurred during the vegetative growth stage (Liu et al. 2019; Sun et al. 2016). The study of Zhou et al. (2018) also demonstrated that higher N inputs in the early stage could reduce low temperature damage to rice, promote tillering recovery and reduce yield

losses caused by low temperatures. Study of N application after low temperature on tillering recovery demonstrated that the tillers increment after low temperature was influenced by the N accumulation increment, that is to say, the higher N accumulation increment, the faster tiller growth (Liu et al. 2019). The same conclusion was obtained in this study: rice grown under a high N supply recovered faster than rice grown under low N supply (Fig. 1). Meanwhile, a synergistic effect between N and Zn emerged after recovery to normal temperature. Increasing Zn application promoted the increase of N accumulation (Fig. 5b) and higher N level also improved Zn absorption (Fig. 3) and transport in rice (Fig. 8), enabling the contribution of Zn in enhancing rice recovery from low temperature. However, the tiller number and shoot Zn concentration presented a quadratic curve relationship at WAR4, meaning that excessive Zn was not still benefit for tiller growth under high N condition and the appreciate shoot Zn concentration was  $32.62 \text{ mg kg}^{-1}$  in this experiment (Fig. 4f). Consequently, Zn could regulate tiller bud germination, promote rice growth recovery under high N levels after recovery to normal temperature by affecting IAA and CTK balance and providing sufficient nutrients for tillering growth through the synergistic effect of N and Zn.

In conclusion, low temperature during the vegetative growth stage damaged the rice antioxidant system, suppressed nutrient uptake and decreased the number of tillers. Zn could enhance rice low temperature resistance and promote tillering recovery from low temperature stress; while the promotion of Zn was related to the environmental N level. Increasing Zn application under suitable N level could diminish the damage of low temperature stress and promote tiller recovery rapidly after low temperature via increasing nutrient absorption and regulating IAA and CTK balance. Increasing Zn application under high N level aggravated the low temperature damage and further reduced tiller number. The inhibition on rice tillering by Zn under high N was mainly due to higher shoot Zn concentration and IAA accumulation, and lower CTK/IAA ratio in tiller buds, which is the main reason lead to tiller dormancy. When the low temperature was relieved, appropriate Zn application under high N level promoted IAA transport from tiller buds, tiller dormancy was broken, grew spurts and then rapidly recovered to normal level.

## Declarations

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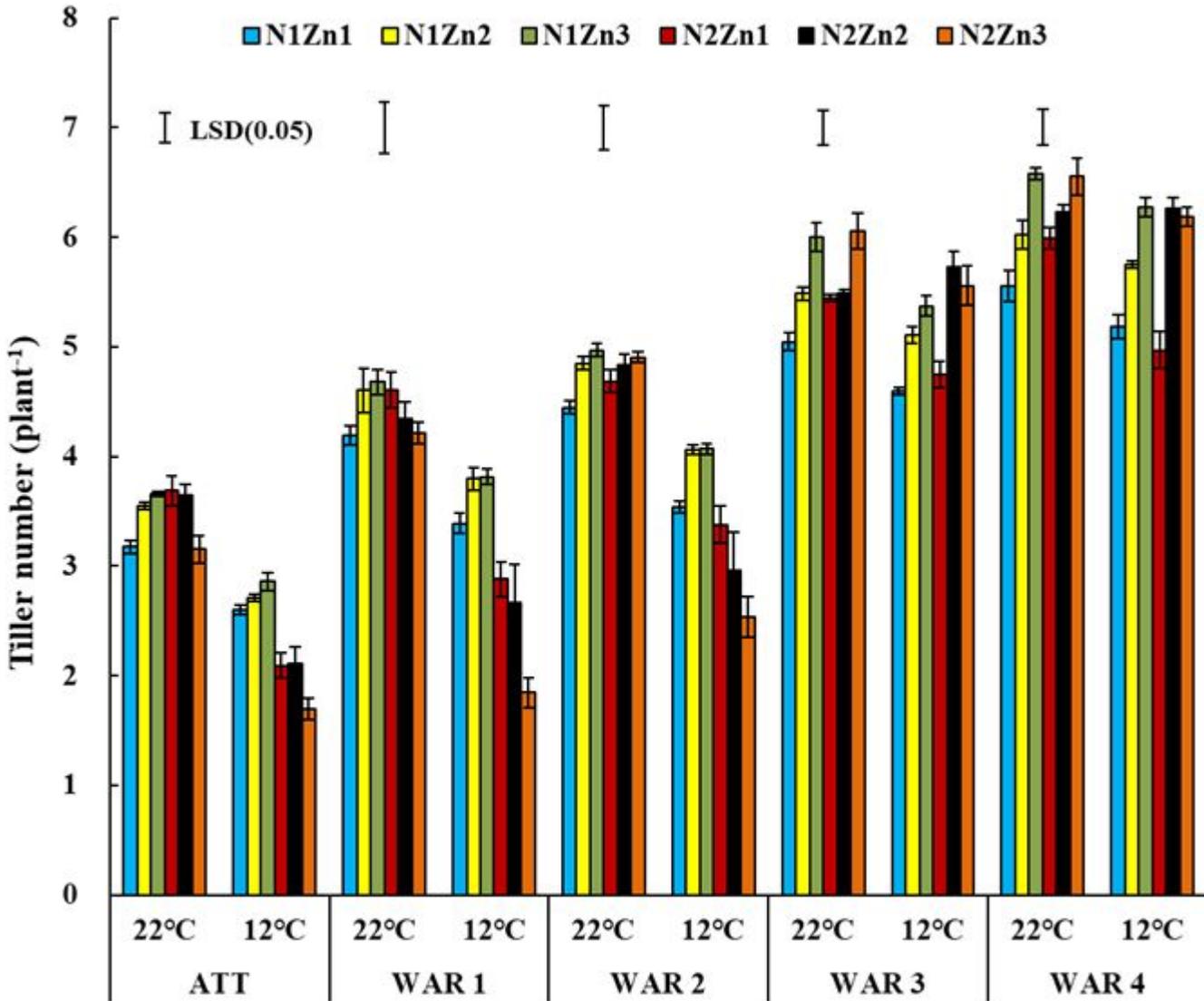
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<https://doi.org/10.1016/j.fcr.2018.1008.1018>

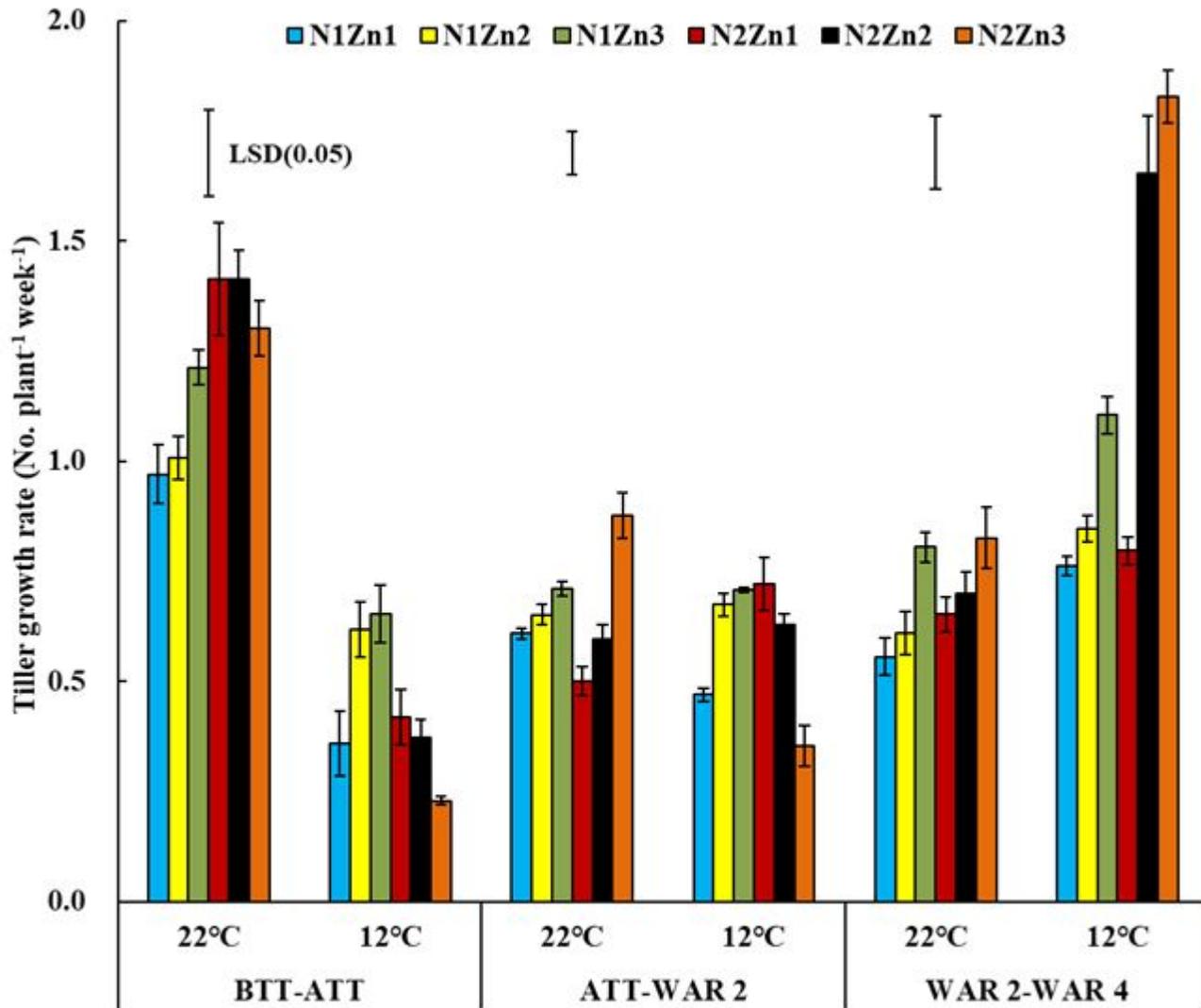
## Figures



**Fig. 1**

Figure 1

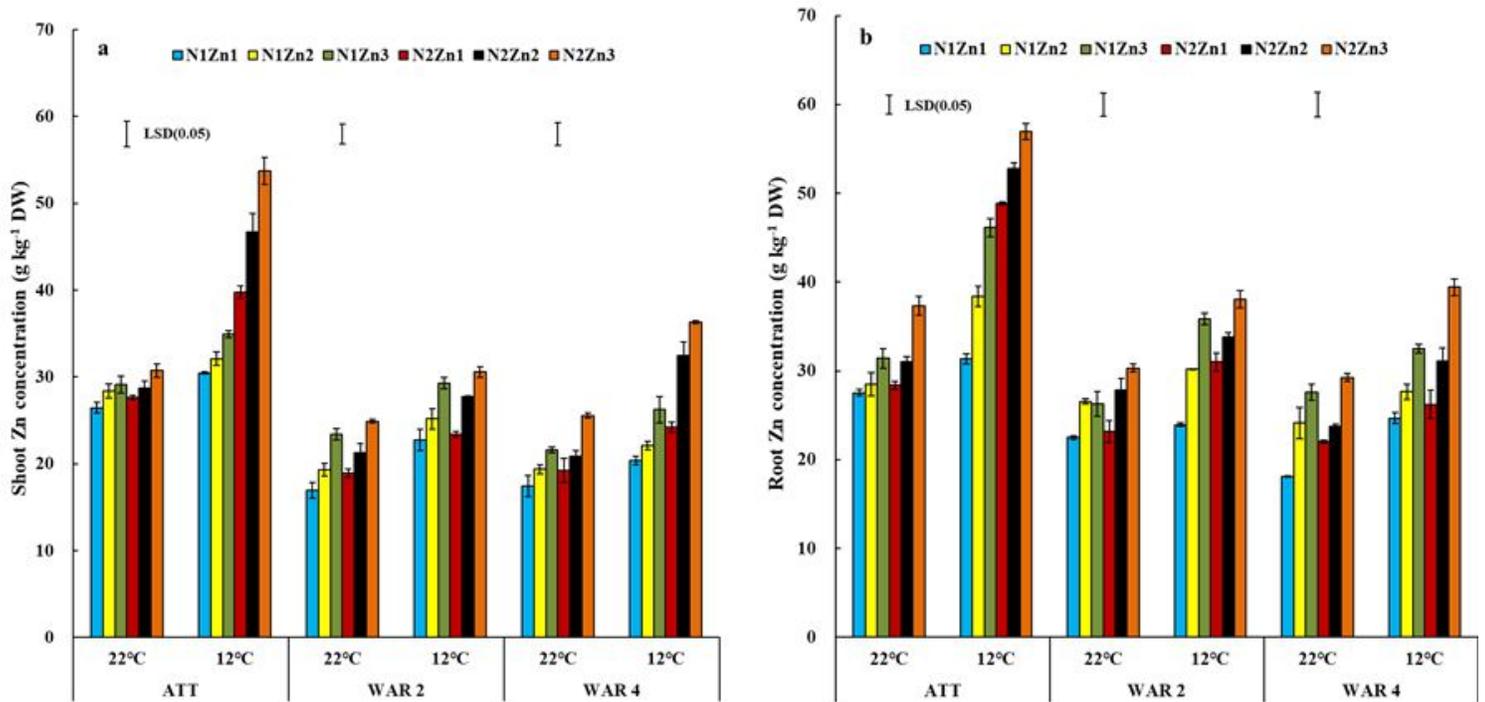
Rice tillering number under different temperature, N and Zn treatments. N1 and N2 represent normal (1.43 mM NH<sub>4</sub>NO<sub>3</sub>) and double solution N (2.86 mM NH<sub>4</sub>NO<sub>3</sub>) level, respectively; Zn1, Zn2 and Zn3 represent half (0.08 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O), normal (0.15 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O) and double solution Zn (0.30 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O) level, respectively. 22°C represents the normal temperature, and 12°C represents the low temperature. ATT represents after temperature treatment, and WAR represents weeks after recovery to normal temperature. Data represent means ± standard errors. from different independent treatments. LSD (0.05) is the least significant difference between treatments at P<0.05.



**Fig. 2**

Figure 2

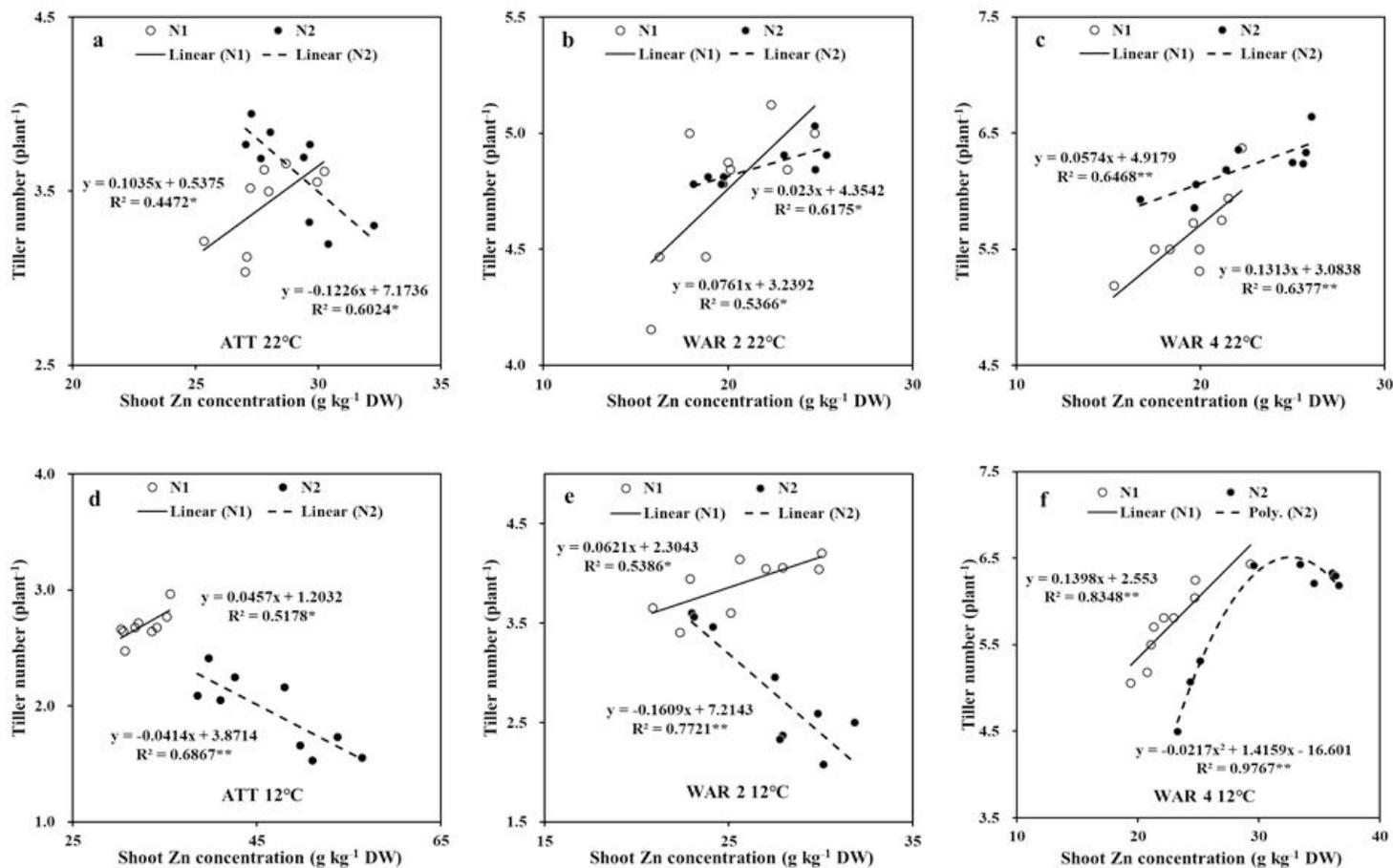
Rice tillering growth rate under different temperature, N and Zn treatments. N1 and N2 represent normal (1.43 mM NH<sub>4</sub>NO<sub>3</sub>) and double solution N (2.86 mM NH<sub>4</sub>NO<sub>3</sub>) level, respectively; Zn1, Zn2 and Zn3 represent half (0.08 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O), normal (0.15 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O) and double solution Zn (0.30 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O) level, respectively. 22°C represents the normal temperature, and 12°C represents the low temperature. ATT represents after temperature treatment, and WAR represents weeks after recovery to normal temperature. Data represent means ± standard errors. from different independent treatments. LSD (0.05) is the least significant difference between treatments at P<0.05.



**Fig. 3**

**Figure 3**

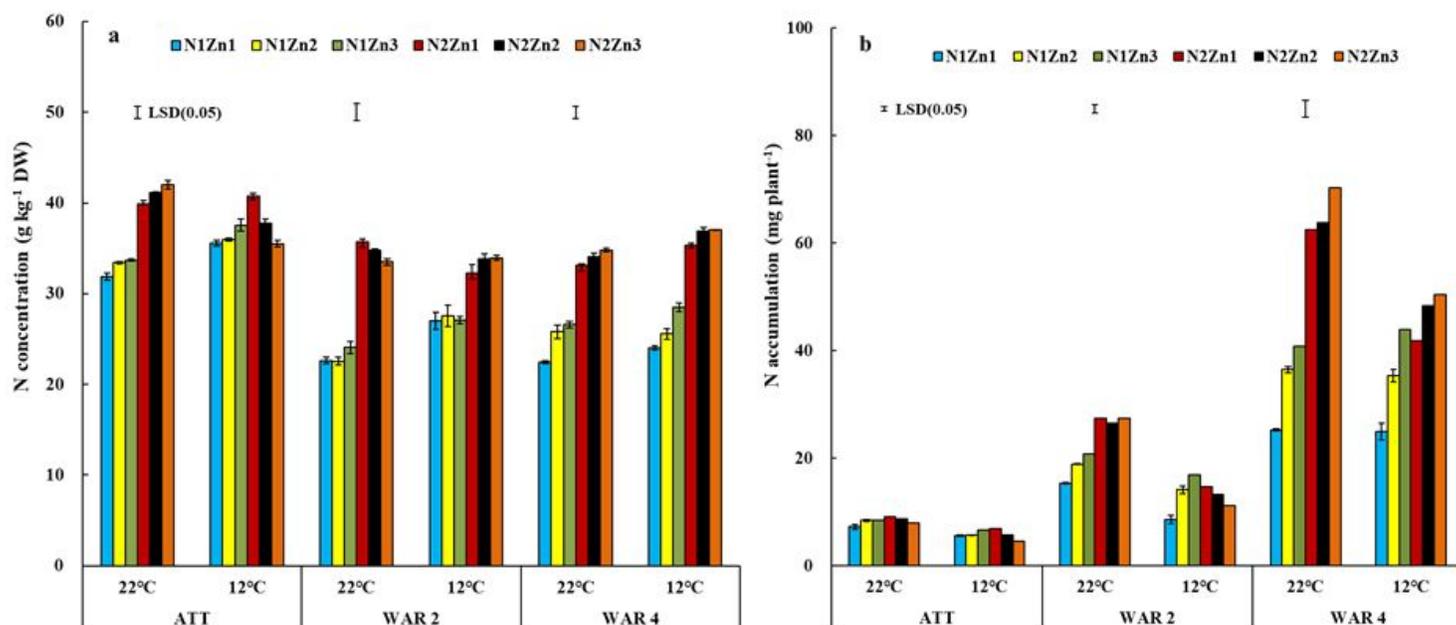
Rice shoot (a) and root (b) Zn concentration under different temperature, N and Zn treatments. N1 and N2 represent normal (1.43 mM NH<sub>4</sub>NO<sub>3</sub>) and double solution N (2.86 mM NH<sub>4</sub>NO<sub>3</sub>) level, respectively; Zn1, Zn2 and Zn3 represent half (0.08 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O), normal (0.15 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O) and double solution Zn (0.30 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O) level, respectively. 22°C represents the normal temperature, and 12°C represents the low temperature. ATT represents after temperature treatment, and WAR represents weeks after recovery to normal temperature. Data represent means ± standard errors. from different independent treatments. LSD (0.05) is the least significant difference between treatments at P<0.05.



**Fig. 4**

**Figure 4**

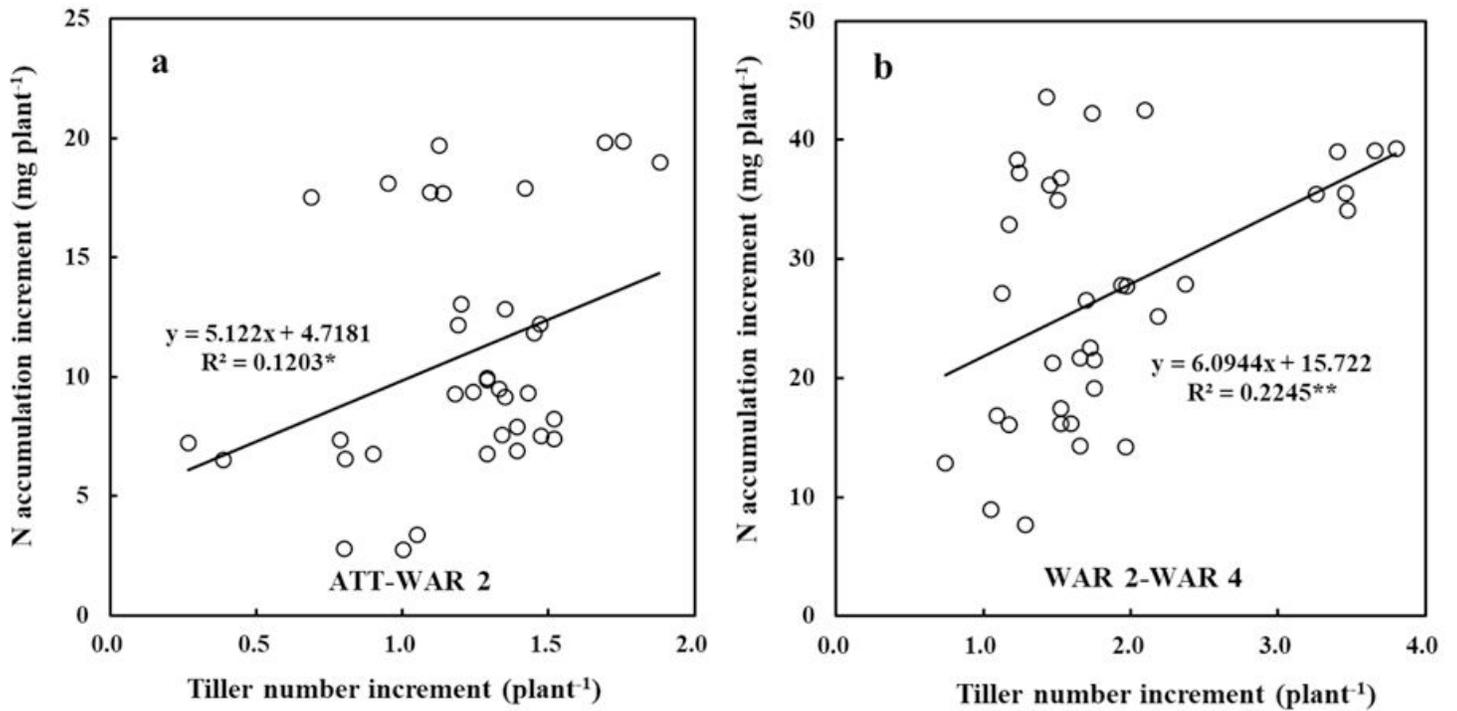
The relationship between shoot Zn concentration and tiller numbers after temperature treatment (ATT) (a and b), 2 weeks after recovery (WAR 2) (c and d) and WAR 4 (e and f) at different N levels, n=9. N1 and N2 represent normal (1.43 mM NH<sub>4</sub>NO<sub>3</sub>) and double solution N (2.86 mM NH<sub>4</sub>NO<sub>3</sub>) level, respectively; Zn1, Zn2 and Zn3 represent half (0.08 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O), normal (0.15 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O) and double solution Zn (0.30 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O) level, respectively. \* and \*\* present significance at P<0.05 and P<0.01, respectively.



**Fig. 5**

**Figure 5**

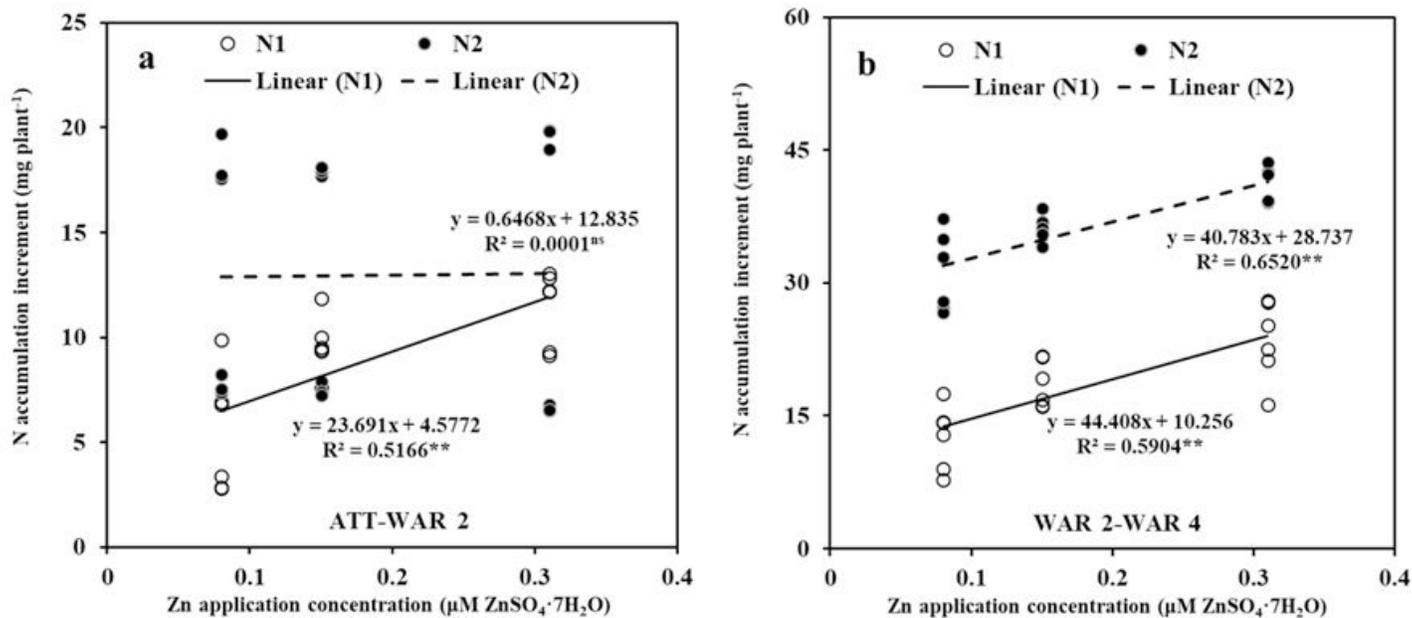
Rice N concentration and N accumulation under different temperature, N and Zn treatments. N1 and N2 represent normal (1.43 mM NH<sub>4</sub>NO<sub>3</sub>) and double solution N (2.86 mM NH<sub>4</sub>NO<sub>3</sub>) level, respectively; Zn1, Zn2 and Zn3 represent half (0.08 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O), normal (0.15 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O) and double solution Zn (0.30 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O) level, respectively. 22°C represents the normal temperature, and 12°C represents the low temperature. ATT represents after temperature treatment, and WAR represents weeks after recovery to normal temperature. Data represent means ± standard errors. from different independent treatments. LSD (0.05) is the least significant difference between treatments at P<0.05.



**Fig. 6**

**Figure 6**

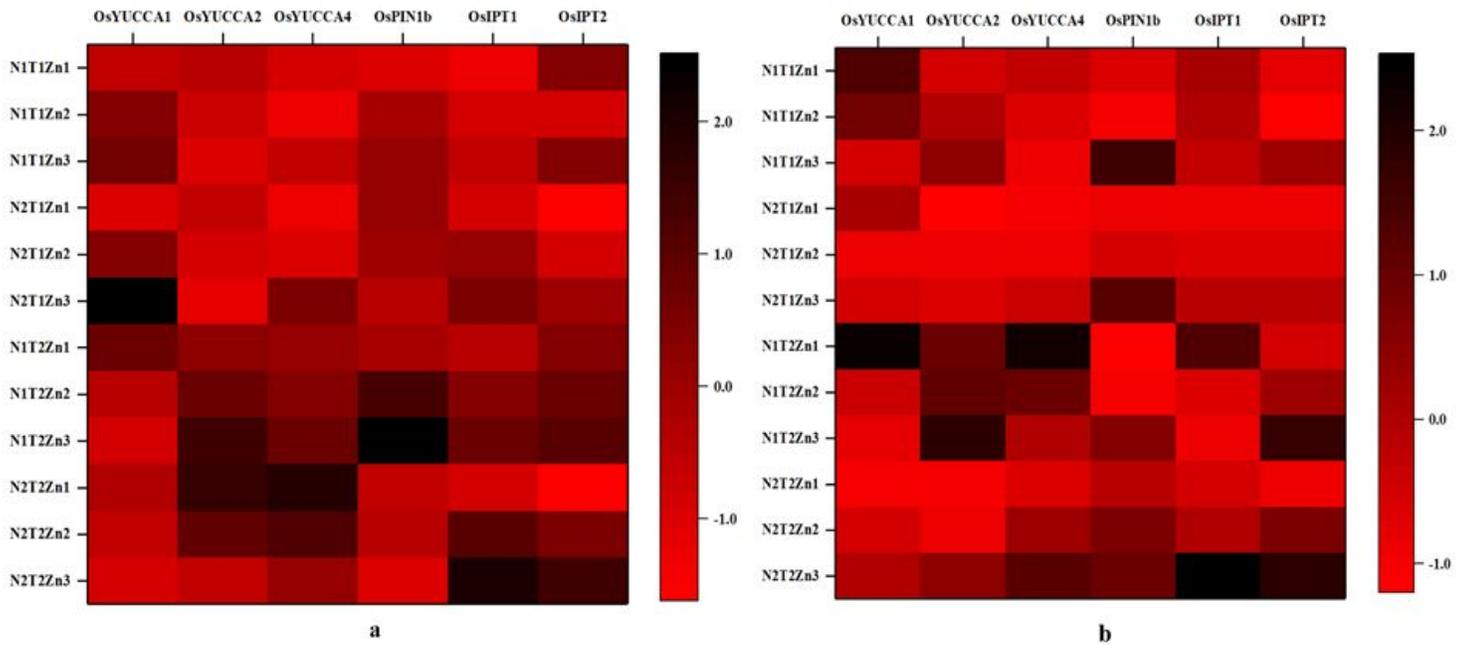
The relationship between the increment of N accumulation and tiller 2 weeks after recovery (WAR 2) (a) and WAR 4 (b). n=36, \* and \*\*present significance at P<0.05 and P<0.01, respectively, and ns means no significance.



**Fig. 7**

**Figure 7**

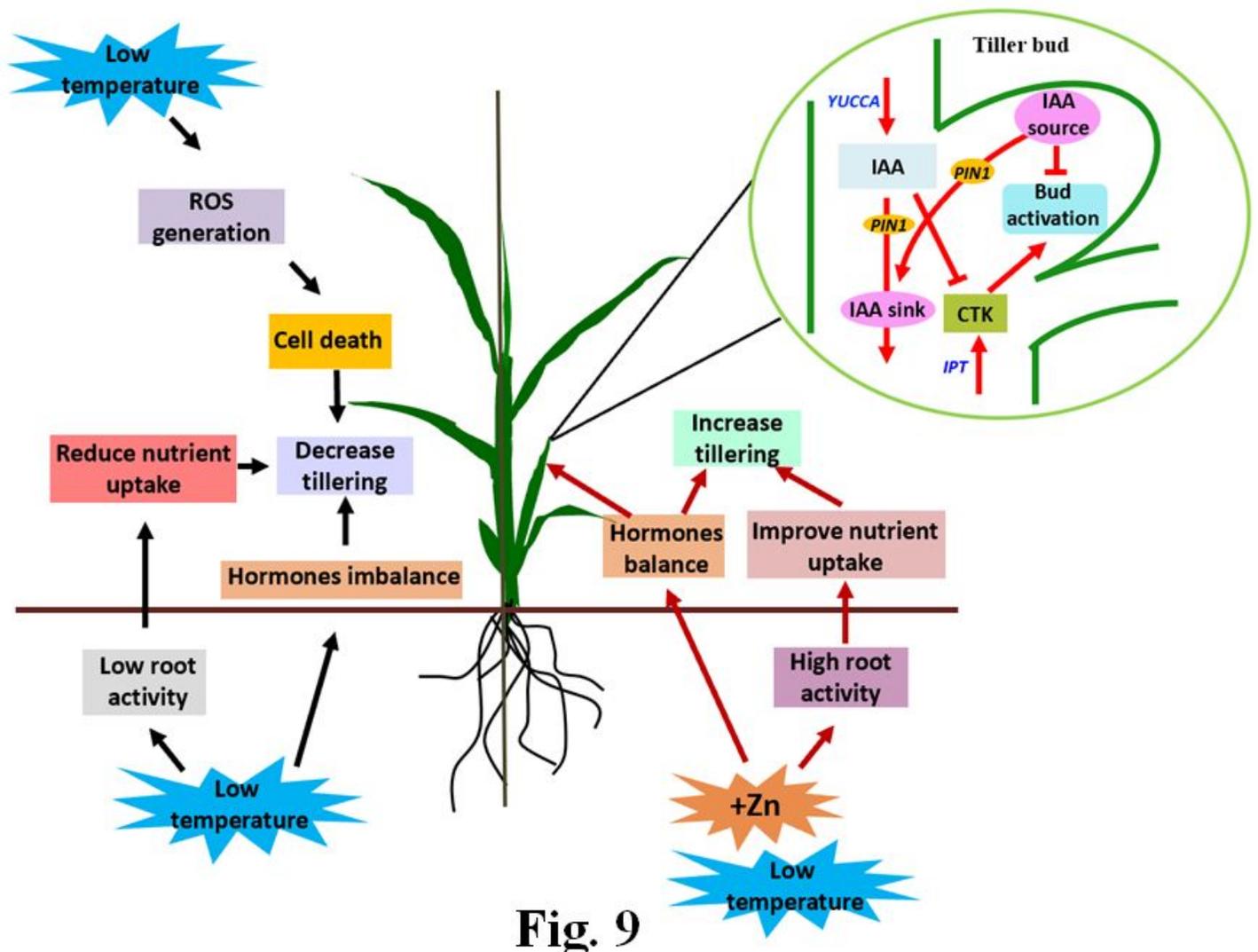
The relationship between Zn concentration and the N accumulation increment 2 weeks after recovery (WAR 2) (a) and WAR 4 (b) at different N levels, n=18. N1 and N2 represent normal (1.43 mM NH<sub>4</sub>NO<sub>3</sub>) and double solution N (2.86 mM NH<sub>4</sub>NO<sub>3</sub>) level, respectively; Zn1, Zn2 and Zn3 represent half (0.08 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O), normal (0.15 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O) and double solution Zn (0.30 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O) level, respectively. \* and \*\* present significance at P<0.05 and P<0.01, respectively, and ns means no significance.



**Fig. 8**

**Figure 8**

The expression of IAA and CTK metabolism-related genes at different N and Zn application levels after temperature treatment (ATT) (a) and 2 weeks after recovery (WAR 2) (b). N1 and N2 represent normal (1.43 mM NH<sub>4</sub>NO<sub>3</sub>) and double solution N (2.86 mM NH<sub>4</sub>NO<sub>3</sub>) level, respectively; Zn1, Zn2 and Zn3 represent half (0.08 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O), normal (0.15 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O) and double solution Zn (0.30 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O) level, respectively. Heat map and hierarchical cluster display the fold changes in gene expression. The color bar represents the level of the fold change, whereby the black color represents downregulation and red signifies an upregulation in expression. The name of the corresponding gene is presented on the top of each heat map.



**Fig. 9**

**Figure 9**

Schematic diagram of Zn increasing rice tillering under low temperature stress. Low temperature causes the decline of root activity, inhibits the nutrient uptake, cause the generation of ROS, disturbs hormones balance and then reduces the number of tillers. Increasing or appropriate Zn application under low temperature can enhance root activity, increase nutrient absorption, promote the synthesis of IAA and cytokine CTK, promote the transport of IAA from tiller bud to other parts, then maintain tiller bud hormones balance, accelerate tillers growth. Meanwhile, Zn can also alleviate the damage of ROS under low temperature stress. ROS, reactive oxygen species; IAA, auxin; CTK, cytokinin; YUCCA, IPT and PIN1, key genes for IAA and CTK synthesis and transport; black arrow, the damage of low temperature to rice; red arrow, the effect of Zn under low temperature stress; green circle, hormones status in tiller bud.

## Supplementary Files

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