Dephosphorylation of Nitrate Reductase Protein Regulates Growth of Rice and Adaptability to Low Temperature

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Abstract

Background: Nitrate reductase (NR) is an important enzyme for nitrate assimilation in plants, and its activity is regulated by post-translational phosphorylation. The change of nitrogen uptake affects the response of rice to low temperature and its growth. To investigate the effect of NIA1 protein dephosphorylation on the growth of rice and its adaptability to low temperature, we analyzed phenotype, chlorophyll content, nitrogen utilization, and antioxidant capacity at low temperature in lines with a mutated NIA1 phosphorylation site (*S532D* and *S532A*), an *OsNia1* over-expression line (*OE*), and wild-type Kitaake rice (*WT*).

Results: Plant height, dry matter weight, and chlorophyll content of *S532D* and *S532A* were lower than those of *WT* and *OE* under normal growth conditions but were higher than those of *WT* and *OE* at low temperature. Compared with *WT* and *OE*, the nitrite, H_2O_2 , and MDA contents of *S532D* and *S532A* leaves were higher under normal growth conditions. The difference in leaf nitrite content between transgenic lines and *WT* was narrower at low temperature, especially in *S532D* and *S532A*, while H_2O_2 and MDA contents of *S532D* and *S532A* leaves were lower than those in *WT* and *OE* leaves. The $\text{NH}_4^+\text{-N}$ and amino acid contents of *S532D* and *S532A* leaves were higher than those of *WT* and *OE* leaves under normal or low temperature. qRT-PCR results revealed that transcription levels of *OsNrt2.4*, *OsNia2*, and *OsNADH-GOGAT* were positively correlated with those of *OsNia1*, and the transcription levels of *OsNrt2.4*, *OsNia2*, and *OsNADH-GOGAT* were significantly higher in transgenic lines than in *WT* under both normal and low temperature.

Conclusions: Phosphorylation of NR is a steady-state regulatory mechanism of nitrogen metabolism, and dephosphorylation of NIA1 protein improved NR activity and nitrogen utilization efficiency in rice. Excessive accumulation of nitrite under normal growth conditions inhibits the growth of rice; however, accumulation of nitrite is reduced at low temperature, enhancing the cold tolerance of rice. These results provide a new insight for improving cold tolerance of rice.

Background

Rice (*Oryza sativa* L.) is a thermophilic crop widely cultivated in most areas of China. Low temperature causes retardation of rice growth and development, and reduction in tillering and fertility, which eventually affect rice yield. Nitrogen is one of the most important and active nutrient factors affecting plant growth and yield (Mi et al. 2017). Studies on the interaction between cold stress and nitrogen fertilizer showed that applying nitrogen fertilizer significantly improves the resistance of rice to low temperature, but high nitrogen levels aggravate the damage caused by low temperature (Cao et al. 2018). Abscissic acid and indole acetic acid levels in rice leaves are improved after treatment with high nitrogen and low temperature (Cai, 2014). Higher abscissic acid and cytokinin content and lower gibberellic acid content slow down the cold injury of plants (Li et al. 2015). Excessive application of nitrogen fertilizer results in tender stems and leaves of rice and susceptibility to cold damage (Cao et al. 2020). Microspore sterility caused by low temperature is aggravated by high nitrogen application during the panicle

primordium differentiation stage of rice, reducing seed setting rate and yield (Hayashi et al. 2000; Hayashi et al. 2006). In production years with occurrence of low-temperature chilling injury, the amount of water and fertilizer applied affects the yield of rice; application of large amounts of nitrogen fertilizer causes especially serious panicle neck phenomenon in the field, and the setting rate and yield of rice panicles is obviously decreased. Therefore, regulation of nitrogen uptake and utilization efficiency affects the tolerance of rice to cold stress, and rice cold tolerance is not consistent under different environmental and nitrogen supply conditions. There are few studies on improving nitrogen assimilation and utilization of rice in response to low temperature stress by molecular methods. Such research might provide a means of enhancing the cold tolerance of rice.

Nitrate reductase (NR) is a rate-limiting enzyme for nitrogen assimilation in higher plants that directly regulates the reduction of nitrate (Crawford 1995). NR activity in plants is inhibited by phosphorylation at a conserved serine residue (Ser, S) in hinge I when plants are transferred from light to darkness (Muslin et al. 1996; Nemie-Feyissa et al. 2013) and ultimately affects plant growth and development (Steven et al. 1996; Kaiser et al. 1999; Harris et al. 2000). Genetic transformation was used to study the regulation of NR activity in tobacco (*Nicotiana plumbaginifolia* Viv.) by fusing the coding region of the *Nia* gene with a promoter driving constitutive expression, thereby relieving transcriptional control of NR. Residues at the phosphorylation site Ser521 were also mutated to aspartic acid residues (Asp, D) and were not phosphorylated (Bachmann et al. 1996; Lea et al. 2006). Release of transcriptional-level control had no significant effect on nitrogen metabolism, while removal of post-translational modification had a profound effect. Wild-type NR was rapidly inactivated when plants were moved from light to dark, while NR with mutated phosphorylation site (S521D) showed constitutive activity (Lillo et al. 2003). The phosphorylation sites of NR are serine residues at amino acid (aa) positions 534, 543, 521, and 528 in *Arabidopsis* (*Arabidopsis thaliana* L.), spinach (*Spinacia oleracea* L.), tobacco (*Nicotiana plumbaginifolia* Viv.), and potato (*Solanum tuberosum* L.), respectively (Bachmann et al. 1996; Steven et al. 1996; Harris et al. 2000; Lea et al. 2006). Through comparative analysis of amino acid sequences, we speculated that phosphorylation of Ser at aa 532 of NIA1 might regulate NR activity in rice. On this basis, we constructed rice lines with a mutated NIA1 phosphorylation site (*S532D* and *S532A*). Phosphorylation level tests revealed that the exogenous NIA1 proteins could not be phosphorylated, and the expression of endogenous NIA1 protein was inhibited (Figure S1).

Studies on the phosphorylation of NR protein have mainly focused on the influence of nucleotide sequence changes on phosphorylation level and the regulation of NR activity by phosphorylation modification. We therefore investigated the effect of deactivating NIA1 phosphorylation on NR activity, nitrogen metabolism, reactive oxygen metabolism, and plant growth in rice using lines with a mutated NIA1 phosphorylation site (*S532D* and *S532A*), an *OsNia1* over-expression line (*OE*), and wild-type Kitaake (WT) at low temperature. Our findings suggest novel methods for improving the cold tolerance of rice by regulating nitrogen metabolism.

Results

Dephosphorylation of NIA1 protein enhances the rice tolerance to low temperature

We observed that growth of lines with a mutated NIA1 phosphorylation site was weaker than that of WT at normal temperature (26 °C), but the situation was opposite at low temperature (8 °C). We hypothesized that phosphorylation of NIA1 protein might affect the stress response of rice to low temperature. To test our hypothesis, we performed a low-temperature test for WT, *S532D*, *S532A*, and *OE* (Fig. 1). Rice seedlings were treated with low temperature at 20 d old, before the different lines showed any major differences in phenotype. Rice seedlings were transferred to low-temperature treatment at 8 °C for 6 d, then transferred back to normal temperature (26 °C) for 3 d; controls were maintained at 26 °C. Plant height and dry matter weight of *S532D* and *S532A* plants were lower than those of WT and *OE* at normal temperature (NT6d and NT9d), while these two indicators were higher in *S532D* and *S532A* than in WT after low-temperature treatment for 6 d followed by normal-temperature treatment for 3 d (LTR3d) (Fig. 1B and C). Except in *S532A*, chlorophyll content remained the same at NT6d as before treatment (BT), being lower in *S532D*, *S532A*, and *OE* than in WT at NT9d (Fig. 1D). Compared with levels at NT6d, chlorophyll contents in all test lines were lower at LT6d, although levels in *S532D* and *S532A* were significantly ($P < 0.05$) higher than those in WT and *OE*. The chlorophyll content of all lines was increased at LTR3d compared with levels at LT6d, and was still significantly higher in *S532D* and *S532A* plants than in WT and *OE* plants. In general, *S532D* and *S532A* showed decreased growth capacity under normal conditions and increased cold tolerance under low temperature, indicating that regulating the dephosphorylation of NIA1 protein might change the stress response of rice seedlings to low temperature.

Dephosphorylation of NIA1 protein increases the ratio of active NR protein

To investigate the effect of low temperature on NR phosphorylation, we assessed NIA1 protein and its phosphorylation levels in WT, *S532D*, *S532A*, and *OE* using immunoblotting with antibodies against NR and phospho-NR (p-NR) (Fig. 2). We adjusted the soluble protein concentration to similar levels in samples from the same line at different time points under low-temperature treatment. Protein analysis results showed that only exogenous OsNIA1 protein expression was detected in *S532D*, *S532A* and *OE*, indicating that endogenous OsNIA1 protein expression was inhibited. Differences in NR phosphorylation level were analyzed in transgenic lines during low-temperature treatment. The specific value of phosphorylated NR to non-phosphorylated NR protein (p-NR/NR) represents the proportion of NR protein that was inactive. The lower the specific value, the higher the activity of NR protein. The specific value of WT was lower at low temperature than at normal temperature. The ratio of p-NR/NR for *S532D* and *S532A* was always 0, indicating that the exogenous NR protein could not be phosphorylated in these two lines due to the mutation of S532 to Asp or Ala, and the NR protein was always in a highly active state. Similar to WT, *OE* also showed high activity of NR at low temperature and low activity at room temperature, and its exogenous NR protein activity was regulated by phosphorylation modification.

Dephosphorylation of NIA1 protein increases NR activity in rice leaves

We observed different stress responses of the test materials to low temperature, which we speculated to be due to the regulation of NR activity by low temperature. As shown in Fig. 3, NR activity was decreased under low temperature, but the reduction in NR activity was smaller in the lines with a mutated NIA1 phosphorylation site (*S532D* and *S532A*) than in the other lines. The NR activity in *S532D* and *S532A* was higher than that in WT or *OE* under normal or low temperature, with WT having the lowest NR activity. Significant differences ($P < 0.05$) in NR activity were caused by replacement of nutrient solutions with fresh nutrients at normal temperature. However, since nutrient solutions were all replaced at the same time, the impact of this on data analysis was relatively small. After returning to normal temperature for 3 d, the NR activity in all lines subjected to low-temperature treatment was much higher than that in plants kept under normal temperature for 9 d.

Dephosphorylation Of Nia1 Protein Increases Nitrogen Utilization In Rice

As can be seen from Fig. 4A, the content of nitrate in leaves of *S532D*, *S532A*, and *OE* was lower than that in WT under normal or low temperature. Nitrate contents after low-temperature treatment for 6 d were higher than those during treatment periods at normal temperature. After normal-temperature recovery growth for 3 d, the content of nitrate increased in leaves of WT and *OE* plants and decreased in those of *S532D* and *S532A*.

According to Fig. 4B-D, contents of ammonium, amino acids, and soluble protein in *S532D* and *S532A* were higher than those in WT and *OE* under normal or low temperature, with WT having the lowest contents. After 6 d of low temperature, the ammonium and amino acid contents of leaves of all lines increased, while the contents decreased again after normal-temperature recovery growth for 3 d.

Differences in nitrite accumulation between transgenic lines and WT decreased at low temperature

Nitrite is the direct product of nitrate reduction by NR. Nitrite must be reduced immediately after its uptake because of its high toxicity to plants (Hachiya et al. 2016). The nitrite content in WT and *OE* was lower at 22:00 pm than at 10:00 am (Fig. 5), which may be due to decreased NR activity at night. However, *S532D* and *S532A* showed the opposite trend, with nitrite content much higher at 22:00 pm than 10:00 am. This indicated that the nitrite reduction rate at night is lower than that during the daytime, and an increase in NR activity leads to rapid accumulation of nitrite at night. The content of nitrite in leaves of *S532D*, *S532A*, and *OE* was approximately one fold higher than that in WT leaves at 10:00 am under normal temperature, while it was 6.1, 6.3, and 1.5 times higher, respectively, at 22:00 pm.

Compared with normal-temperature treatment for 6 d, the content of nitrite in leaves of *S532D*, *S532A*, and *OE* was significantly lower after low-temperature treatment for 6 d, while the WT nitrite content remained similar. The content of nitrite in leaves of *S532D*, *S532A*, and *OE* was 0.59, 0.31, and 0.35 times greater, respectively, than that in WT at 10:00 am under low-temperature treatment, while it was 1.2, 1.3, and 0.19 times greater, respectively, at 22:00 pm. When normal temperature growth resumed for 3 d, the

nitrite content of *S532D* and *S532A* increased, especially at 22:00 pm. Compared with WT, the content of nitrite in leaves of *S532D*, *S532A*, and *OE* was 0.42, 0.44, and 0.08 times higher, respectively, at 10:00 am, while it was 3.4, 2.9, and 0.24 times higher at 22:00 pm, respectively. This indicated that low temperature can reduce the accumulation of nitrite, narrowing the gap between transgenic lines and WT.

H₂O₂ and MDA contents of transgenic lines decrease at low temperature

Contents of H₂O₂ and MDA in *S532D*, *S532A*, and *OE* were significantly higher than those in WT under normal temperature (Fig. 6A and B). H₂O₂ and MDA contents of all lines increased after low-temperature treatment for 6 d, but decreased again after normal-temperature recovery growth for 3 d. Contents of H₂O₂ and MDA in leaves of *S532D* and *S532A* were lower than those in leaves of WT and *OE* after low-temperature treatment for 6 d and recovery for 3 d. These results showed that the antioxidant capacity of *S532D* and *S532A* at normal temperature was lower than that of WT and *OE*, while the antioxidant capacity of *S532D* and *S532A* at low temperature was higher than that of WT and *OE*, improving cold tolerance.

Transcript levels of genes involved in nitrogen catabolism upstream and downstream of *OsNia1*

Two *Nia1* genes have been identified in rice, designated as *OsNia1* and *OsNia2*, respectively (Chen et al. 2013). *OsNRT2.4* is a dual-affinity transporter that functions in nitrate-regulated root growth and nitrate distribution in rice (Wei et al. 2018). Nitrite reductase (NiR) is the control enzyme for plant nitrite (NO₂⁻) assimilation (Wang et al. 2007). Glutamic acid is one of the basic amino acids of nitrogen metabolism in organism; ammonium is reduced to glutamate (Glu) by glutamine synthetase (GS)-glutamate synthase (GOGAT) pathway enzymes, and Glu serves as the precursor for the amino acid cycle (Sathee et al. 2021). Insertion of exogenous *OsNia1* or mutant *OsNia1* genes into rice produced significantly higher transcript levels of *OsNia1* in *S532D*, *S532A*, and *OE* than in WT (Fig. 7A and B). Transcript levels of the NR gene (*OsNia2*), nitrate transporter gene (*OsNrt2.4*), NiR gene (*OsNiR*), and glutamate synthase gene (*OsNADH-GOGAT*) in these transgenic lines all increased as *OsNia1* expression level increased at normal temperature. Transcript levels of *OsNia2*, *OsNrt2.4*, and *OsNADH-GOGAT* also increased while those of *OsNiR* decreased as *OsNia1* expression increased at low temperature. Correlation analysis of these gene transcript levels showed that except for the extremely significant negative correlation between the transcript levels of *OsNiR* and *OsNia1* under low-temperature treatment for 4 d, expression of all other genes was positively correlated with that of *OsNia1* throughout the experiment (Fig. 7C).

Discussion

Low temperature reduces nitrite accumulation in rice to reduce growth inhibition

We observed that rice seedlings of *S532D* and *S532A* showed weaker growth than those of WT and *OE* at normal temperature, while the situation was reversed under low temperature. Plant height, dry weight, and

chlorophyll content of *S532D* and *S532A* were lower than those of WT and *OE* at normal temperature, but higher than those of WT and *OE* following low-temperature treatment for 6 d and normal-temperature recovery for 3 d (Fig. 1). Meanwhile, H_2O_2 and MDA contents of *S532D* and *S532A* were higher than those of WT and *OE* at normal temperature. H_2O_2 and MDA are metabolic substances produced by plants in response to adverse environmental conditions. According to the biological radical injury theory, damage is caused by an imbalance between production and elimination of free radicals in cells under stress; excessive reactive oxygen species can lead to membrane lipid peroxidation, which causes accumulation of MDA and affects the normal growth of plants (Mchdy 1994; Gill and Tuteja 2010). However, the difference between our transgenic lines and WT mainly lies in the activity of NR and the accumulation of nitrogen metabolites (Figs. 2 and 3). Previous studies have shown that excessive accumulation of nitrite and ammonium in plants is self-damaging (Britto et al. 2002; Lillo et al. 2003; Lea et al. 2004). In this study, the nutrient solution used in the test contained 20 mg/L NO_3^- -N and 20 mg/L NH_4^+ -N. Excessive transformation of NO_3^- -N was the reason for weak growth of *S532D* and *S532A*. Analysis of nitrogen utilization showed that *S532D* and *S532A* could efficiently utilize NO_3^- -N to generate NH_4^+ -N and amino acids, while excessive accumulation of nitrite and ammonium occurred in leaves (Figs. 3B and 4). The nitrite content in *S532D* and *S532A* was much higher than that in *OE* and WT at normal temperature, especially at night. Meanwhile, the content of nitrite in *S532D* and *S532A* decreased significantly under low temperature, and the ammonium content of *S532D* and *S532A* was higher than that of WT or *OE*. Low temperature promoted the accumulation of ammonium in rice leaves, and the ammonium content in *S532D* and *S532A* was higher than that in WT and *OE*. Studies of tobacco NR protein phosphorylation showed that an increase in NR activity leads to excessive accumulation of nitrite in the dark, which might cause tobacco S521 seedlings to grow weakly (Lillo et al. 2003). This indicates that the weak growth of *S532D* and *S532A* was mainly due to the accumulation of nitrite at normal temperature. Meanwhile, as NR activity decreased, the accumulation of nitrite decreased accordingly during low-temperature treatment. In addition, the difference in nitrite content between transgenic lines and WT was reduced under low temperature, decreasing the damage to *S532D* and *S532A* caused by nitrite. The accumulation and reduction of nitrite is quite rapid, and the key enzyme involved in this process is nitrite reductase (NiR). Studies have found that there is coupling regulation between NR and NiR, and NiR activity has a similar circadian rhythm to NR activity in plants (Faure et al. 1991). Our qRT-PCR results showed that the transcription level of *OsNiR* was positively correlated with that of *OsNia1* at normal temperature (Fig. 7). These results indicate that despite increased transcriptional level or enzyme activity of NiR, increased NR enzyme activity still disrupted the balance between the production and reduction of nitrite. Expression of *OsNiR* was significantly negatively correlated with that of *OsNia1* at low temperature. However, compared with those at normal temperature, the contents of nitrite in *S532D* and *S532A* leaves were significantly lower under low temperature. The rate of nitrite biosynthesis was therefore lower than the rate of nitrite reduction. In general, the forced change in NR activity and low temperature broke the synergistic regulation of NR and NiR.

Dephosphorylation of NIA1 protein enhances nitrogen utilization and plant growth in rice at low temperature

Previous studies have demonstrated that S532 is an important phosphorylation site of NIA1 protein and that directional mutation of phosphorylation site S532 improves NR activity and the assimilation of nitrate in rice (Han et al. 2020). The same result is shown in Figs. 2, 3, and 4; NR activity and the nitrate assimilation rate of lines with a mutated NIA1 phosphorylation site were enhanced under low or normal temperature, while low temperature inhibited NR activity and increased nitrate nitrogen, ammonium nitrogen, and free amino acid contents in all lines tested. All phenotypic indicators of *S532D* and *S532A*, including plant height, dry matter weight, and chlorophyll content, were higher than those of WT and *OE* at low temperature, while the contents of H_2O_2 and MDA in *S532D* and *S532A* were lower than those in WT and *OE*, indicating that increased NR activity enhances the tolerance of rice to low-temperature stress. The utilization efficiency of NO_3^- -N in *S532D* and *S532A* during low-temperature treatment was much higher than that in WT and *OE*, and the accumulation of amino acids in the leaves was also higher, enhancing the ability of rice to resist low-temperature stress. Amino acids are the building blocks of proteins. Low temperature promotes the accumulation of soluble protein, and cold-tolerant varieties have higher soluble protein content (Chung et al. 2006; Deng et al. 2011; Sun et al. 2014). Meanwhile, our qRT-PCR results showed that transcription levels of *OsNia2*, *OsNrt2.4*, and *OsNADH-GOGAT* were positively correlated with those of *OsNia1*, indicating that an increase in NR activity improves nitrate transport efficiency and ammonium assimilation efficiency. In general, *S532D* and *S532A* had higher nutrient levels and stronger antioxidant capacity than WT at low temperature.

Conclusion

Phosphorylation of NIA1 protein can effectively change NR activity and regulate nitrogen utilization, making it a hotspot of NR research. We determined that the S532 phosphorylation site of NIA1 is important in rice, regulating the circadian rhythm of NR, and mutation of S532 confers a stable active state on NR. At normal temperature, directed mutation of NIA1 S532 intensifies the toxicity of nitrite accumulation in rice leaves. Under low temperature, however, directed mutation of NIA1 S532 improves the assimilation ability of nitrate to meet the plants' nutritional needs, reduces the accumulation of nitrite, and enhances the tolerance of rice to low temperature. This study on the regulatory mechanisms of NR phosphorylation not only provides new resources for introducing efficient utilization of nitrogen and cold tolerance in rice, but also provides new clues for the physiological mechanism of NR phosphorylation regulating rice growth and development.

Materials And Methods

Plant material and growth conditions

Rice lines with a mutated NIA1 phosphorylation site (*S532D* and *S532A*), an *OsNia1* over-expression line (*OE*), and wild-type Kitaake (WT) were used. *S532D* and *S532A* were obtained by transferring exogenous *OsNia1* (LOC4345795) with Ser-532 mutated into Asp or alanine (Ala, A) into Kitaake; exogenous OsNIA1 protein was the fusion protein of endogenous NIA1 and GFP protein (28KD), exogenous OsNIA1 expressed from either construct could not be phosphorylated, endogenous OsNIA1 protein expression

was inhibited and its protein level could not be detected (Figure S1B). *OE* plants were similar to *S532D* and *S532A* with the exception that the rice NIA1 protein was intact. In *S532D*, *S532A*, and *OE*, exogenous NR was constitutively expressed using the *CamV35S* promoter. All transgenic lines used in this study were homozygous T3 plants. Construction of the carrier and genetic transformation of vectors were completed in the laboratory of Wan Jianmin, Institute of Crop Science, Chinese Academy of Agricultural Sciences.

Experiments were performed at the Key Laboratory of Crop Physiology, Ecology, and Genetics Breeding of Jiangxi Agricultural University, Nanchang, Jiangxi, P.R. China. Test materials were grown in a walk-in climate-controlled chamber. Seeds were sterilized for 15 min using 1% (v/v) formalin solution (40%, v/v, formaldehyde) and allowed to germinate for 3 d; well-germinated seeds were then placed into individual wells of 96-well PCR plates. Seedlings were cultured in hydroponic nutrient solution formulated according to the formula of the International Rice Research Institute (IRRI) (114.3 mg/L NH_4NO_3 , 50.4 mg/L $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 89.3 mg/L K_2SO_4 , 158.2 mg/L CaCl_2 , 405 mg/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.875 mg/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 92.5 $\mu\text{g/L}$ $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 2\text{H}_2\text{O}$, 1.1675 mg/L H_3BO_3 , 43.75 $\mu\text{g/L}$ $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 38.75 $\mu\text{g/L}$ $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 97.125 mg/L $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 14.875 mg/L citric acid). Growth conditions were 12 h/12 h (day/night), 28 °C/26 °C (day/night), 600/0 $\mu\text{mol/m}^2/\text{s}$ (day/night) photosynthetic photon flux density, and 75% relative humidity; nutrient solution was replaced every 3 days. Rice seedlings grown for 20 d were transferred to low-temperature treatment at 8 °C for 6 d, then transferred back to normal temperature (26 °C) for recovery growth for 3 d; the control was cultured at normal temperature throughout, and three replications were used for low-temperature treatment. Rice leaves were collected at 10:00 and 22:00 h before low-temperature treatment (BT), after low-temperature treatment for 2 d (LT 2d), 4 d (LT 4d), and 6 d (LT 6d), and after normal-temperature treatment for 3 d following low-temperature treatment for 6 d (LTR 3d), respectively. Two rows (16 plants) were randomly mixed for each sampling, and six repeat samples were obtained for storage at -80 °C.

Levels of NIA1 protein and phosphorylation

Fresh leaves (0.5 g) were ground into a powder in liquid nitrogen, homogenized with 1.5 mL of protein extract (50 mM Tris·HCl, 150 mM NaCl, 10 mM MgCl_2 , 1 mM EDTA, 10% (v/v) glycerol, 20 mM NaF, 50 mM DTT, 10 mM PMSF), and centrifuged at 12000 rpm (10200 *g*) for 10 min. The supernatant was retained as the protein extract. Soluble protein content in protein extract was determined using Coomassie bright blue colorimetry (Han et al. 2018). The protein concentrations were adjusted to the same level by adding protein extract, and the same loading volume was used to detect phosphorylated protein as non-phosphorylated protein. SDS (5%) sample loading buffer (sample:buffer = 4:1) was added, and proteins were denatured at 105 °C for 10 min. Immunoblotting was used to analyze the protein content and phosphorylation level of NIA1. Antibodies against the NIA1 protein and its phosphorylated form were prepared by ABclonal Technology Co., LTD (Wuhan, China). Quantitative analyses were performed by scanning formazan bands in the gel using a computing laser densitometer (Molecular Dynamics) using Image J2x software (USA).

NR activity

NR activity was determined according to the method described by Lea et al. (2006). Three samples were tested for each treatment group in each replicate experiment. Leaves (0.5 g) were homogenized with 4 mL of 0.1 M HEPES-KOH, pH 7.5, 3% (w/v) PVP, 1 mM EDTA, and 7 mM Cys. The assay mixture contained 50 mM HEPES-KOH, pH 7.5, 100 mM NADH, and 5 mM KNO₃ with 2 mM EDTA or 6 mM MgCl₂. Assay volume was 2 mL. Activity was measured in crude extracts by determining NO₂⁻ formation following the addition of 1% (w/v) sulfanilamide and 0.2% (w/v) *N*-(1-naphthyl)ethylenediamine dihydrochloride in 3 M HCl. Activity state is defined as NR assayed in the presence of Mg²⁺ (and 14-3-3 proteins) as a percentage of NR activity measured in the presence of EDTA and reflects how much of the enzyme is in the non-phosphorylated active form. Assays were run at 25 °C.

Nitrate, ammonium, free amino acid, and soluble protein contents

Rice leaves (0.5 g) were ground into a powder and added to 10 mL double-distilled water. Nitrate, ammonium, and amino acids were extracted using boiling water for 30 min. Three samples were tested for each treatment group in each replicate experiment. Nitrate content in rice leaves was determined using nitrosalicylic acid colorimetry (Cataldo et al. 1975). Ammonium content in rice leaves was determined using indophenol blue colorimetry (Hachiya et al. 2012). Amino acid content in rice leaves was determined using the ninhydrin method (Xu et al. 2017).

Rice leaves (0.5 g) were ground into a homogenate with 2mL of 50 mmol/L phosphoric acid buffer (pH7.0) (6.8g/L KH₂PO₄, 1.164 g/L NaOH), washed with 6 mL double-distilled water and the sample volume adjusted to 10mL. Three samples were tested for each treatment group in each replicate experiment. Soluble protein content in rice leaves was determined using Coomassie bright blue colorimetry (Han et al. 2018).

Chlorophyll content

Chlorophyll content was determined spectrophotometrically after extraction of shoots with 80 % (v/v) acetone (Lattanzio et al. 2009). Three samples were tested for each treatment group in each replicate experiment.

Nitrite content

Nitrite content was determined according to the method described by Hachiya et al. (2016). Three samples were tested for each treatment group in each replicate experiment. Leaves (0.5 g) were homogenized with 4 mL of 0.1 M HEPES-KOH, pH 7.5, 3% (w/v) PVP, 1 mM EDTA, and 7 mM Cys and centrifuged at 20400 rpm (29500 *g*) for 10 min. The supernatant was retained, and 1 mL 1% (w/v) sulfanilamide and 2 mL 0.2% (w/v) *N*-(1-naphthyl)ethylenediamine dihydrochloride were added to 1 mL extract, mixed, and incubated for 20 min at 23 °C before centrifuging at 4000 rpm (1133 *g*) for 5 min.

Absorbance was determined at 540 nm. Nitrite contents in leaves were calculated according to the regression equation obtained from a standard curve.

Hydrogen peroxide (H₂O₂) and malondialdehyde (MDA) contents

H₂O₂ content was determined using the titanium sulfate method (Qi et al. 2006). MDA content was measured using the thiobarbituric acid (TBA) method (Qi et al. 2006).

Analysis of gene expression related to nitrogen metabolism

Total RNA was isolated from the leaves of seedlings using a TaKaRa MiniBEST Plant RNA Extraction Kit (TaKaRa, China). Approximately 0.5 µg of total RNA was reverse-transcribed to first-strand cDNA using PrimeScript™ RT Master Mix (TaKaRa, China). qRT-PCR detection was performed on a CFX96 Real-Time PCR Detection System using SYBR® Premix Ex Taq TM II (TaKaRa, China). The soybean housekeeping gene *Ubiquitin* (LOC4327162) was used as an internal reference to normalize data. Expression of the following genes related to nitrogen metabolism was determined: NR genes *OsNia1* (LOC4345795) and *OsNia2* (LOC4330867), nitrate transporter protein gene *OsNrt 2.4* (LOC4336175), nitrite reductase gene *OsNiR* (LOC4330835), and glutamate synthase gene *OsNADH-GOGAT* (LOC4324398). All primers were designed using NCBI (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) (Table 1).

Table 1 Primer sequences for qRT-PCR

Primer name	Primer sequence (5'-3')	Target gene
<i>OsNia1</i> -3F	TGGCCGGAAAGTTACAAGGG	<i>OsNia1</i>
<i>OsNia1</i> -3R	CGACATCGACGGACCAGAAG	<i>OsNia1</i>
<i>OsNia2</i> -2F	CTTGGTGGTACAAGCCGGAG	<i>OsNia2</i>
<i>OsNia2</i> -2R	AGCACCAGCACCAGTACTTT	<i>OsNia2</i>
<i>OsNrt2.4</i> -2F	GCGACCATGTCACGGTGATG	<i>OsNrt2.4</i>
<i>OsNrt2.4</i> -2R	GTAGTTCTCGGTGCTTGCCA	<i>OsNrt2.4</i>
<i>OsNADH-GOGAT</i> -F	CTGACATGATTTCTGTGGACG	<i>OsNADH-GOGAT</i>
<i>OsNADH-GOGAT</i> -R	ACGAGAAGGCTGTTCCCCTC	<i>OsNADH-GOGAT</i>
<i>OsNiR</i> -1F	GACATCGACGTGCGTCTCA	<i>OsNiR</i>
<i>OsNiR</i> -1R	CTTGCCGTACGCCTCGATCA	<i>OsNiR</i>
<i>ubiquitin</i> -F	AACCAGCTGAGGCCCAAGA	<i>ubiquitin</i>
<i>ubiquitin</i> -R	ACGATTGATTTAACCAGTCCATGA	<i>ubiquitin</i>

Statistical analysis

Individual means and standard errors of the means were calculated using data from independent samples in Microsoft Excel 2007 (Microsoft, USA). IBM SPSS Statistics 22 (SPSS 22, SPSS Inc, USA) was used for statistical analysis and correlation analysis, and the least significant difference method (LSD) was used to determine significant differences between means. Level of significance was checked at $P < 0.05$ and indicated by different letters. Geneious R10 (Biomatters, New Zealand) was used for protein sequence alignment analysis.

Abbreviations

NR: Nitrate reductase; WT: wild type; *S532D and S532A*: lines with a mutated NIA1 phosphorylation site; *OE*: an *OsXDH* over-expression transgenic line; Ser: serine residue; Asp: aspartic acid residues; NIA1: Nitrate reductase protein; EDTA: Ethylene diamine tetraacetic acid; DTT: DL-Dithiothreitol; PMSF: Phenylmethanesulfonyl fluoride; SDS: Sodium dodecyl sulfate; NADH: Nicotinamide adenine dinucleotide; PVP: Polyvinyl pyrrolidone; Cys: L(+)-Cysteine; qRT-PCR: Quantitative reverse transcription PCR; LSD: The least significant difference method; MDA: malonaldehyde.

Declarations

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Author contributions

RH designed and participated in all experimental procedures, performed data analysis, and drafted the manuscript. CL, YW, QS, XP, HL, HJ, and ZW supervised the study and critically revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. Sequences used in this study are available from the National Center for Biotechnology Information (NCBI).

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Opposing regulation of rice seedling growth at different temperatures. Representative pictures are shown (A), and plant height (B), dry weight (C), and chlorophyll content (D) were measured. WT, wild type. *S532D* and *S532A*, NIA1 phosphorylation site directed mutant lines. *OE*, *OsNia1* over-expression line. BT, before low-temperature treatment (i.e., age of rice seedlings was 20 d). NT6d, normal-temperature (26°C) treatment for 6 d. LT6d, low-temperature treatment for 6 d. NT9d, normal-temperature treatment for 9 d. LTR3d, normal-temperature treatment for 3 d after low-temperature treatment for 6 d. Bar=5 cm(A). Column data are means, and short lines represent mean square deviation; values in the same column show means via the LSD, and different letters indicate that the means were statistically different ($P<0.05$), $n=3$.

Figure 2

NR protein and its phosphorylation level in rice seedlings under low temperature. (A) NR protein and its phosphorylation level, . (B) Specific value (p-NR/NR). Soluble protein was extracted from rice leaves treated at room and low temperature. The level of NR protein in each line was made similar by adjusting the content of soluble protein, and the phosphorylation level of NR was detected using the same amount of soluble protein as non-phosphorylated protein. NR and p-NR were analyzed by immunoblotting with

anti-NR and anti-p-NR antibodies, respectively. The exposure time for observing the imprint was 1 min. Numbers above lanes indicate relative intensity obtained by scanning the formazan bands with a computing laser densitometer using Image J 2x software.

Figure 3

NR activity of rice seedlings under low temperature. WT, wild type. S532D and S532A, NIA1 phosphorylation site directed mutant lines. OE, OsNia1 over-expression line. BT, before low-temperature treatment (i.e., age of rice seedlings was 20 d). NT2d, normal-temperature treatment for 2 d. LT2d, low-temperature treatment for 2 d. NT4d, normal-temperature treatment for 4 d. LT4d, low-temperature treatment for 4 d. NT6d, normal-temperature treatment for 6 d. LT6d, low-temperature treatment for 6 d. NT9d, normal-temperature treatment for 9 d. LTR3d, normal-temperature treatment for 3 d after low-temperature treatment for 6 d. Column data are means, and short lines represent mean square deviation; values in the same column show means via the LSD, and different letters indicate that the means were statistically different (P<0.05), n=3.

Figure 4

Nitrate, ammonium, and amino acid contents of rice seedlings under low temperature. **(A)** Nitrate content. **(B)** Ammonium content. **(C)** Amino acid content. WT, wild type. *S532D* and *S532A*, NIA1 phosphorylation site directed mutant lines. *OE*, *OsNia1* over-expression line. BT, before low-temperature treatment (i.e., age of rice seedlings was 20 d). NT6d, normal-temperature treatment for 6 d. LT6d, low-temperature treatment for 6 d. NT9d, normal-temperature treatment for 9 d. LTR3d, normal-temperature treatment for 3 d after low-temperature treatment for 6 d. Column data are means, and short lines represent mean square deviation; values in the same column show means via the LSD, and different letters indicate that the means were statistically different ($P < 0.05$), $n = 3$.

Figure 5

Nitrite content in rice seedlings under low temperature. WT, wild type. *S532D* and *S532A*, NIA1 phosphorylation site directed mutant lines. *OE*, *OsNia1* over-expression line. BT, before low-temperature treatment (i.e., age of rice seedlings was 20 d). NT6d, normal-temperature treatment for 6 d. NT9d, normal-temperature treatment for 9 d. LT6d, low-temperature treatment for 6 d. LTR3d, normal-temperature treatment for 3 d after low-temperature treatment for 6 d. Column data are means, and short lines represent mean square deviation; values in the same column show means via the LSD, and different letters indicate that the means were statistically different ($P<0.05$), $n=3$.

Figure 6

H₂O₂ and MDA contents in transgenic seedlings under low temperature. **(A)** H₂O₂ content. **(B)** MDA content. WT, wild type. *S532D* and *S532A*, NIA1 phosphorylation site directed mutant lines. *OE*, *OsNia1* over-expression line. BT, before low-temperature treatment (i.e., age of rice seedlings was 20 d). NT6d, normal-temperature treatment for 6 d. NT9d, normal-temperature treatment for 9 d. LT6d, low-temperature treatment for 6 d. LTR3d, normal-temperature treatment for 3 d after low-temperature treatment for 6 d.

Column data are means, and short lines represent mean square deviation; values in the same column show means via the LSD, and different letters indicate that the means were statistically different ($P<0.05$), $n=3$.

Figure 7

Analysis of gene expression related to nitrogen metabolism. (A) Expression analysis of nitrogen metabolism-related genes at normal temperature for 4 d. (B) Expression analysis of nitrogen metabolism-related genes at low temperature for 4 d. Column data are means, and short lines represent mean square deviation, values in the same column show means via the LSD, and different letters indicate that the means were statistically different ($P<0.05$), $n=3$. (C) Correlation analysis of *OsNia1* gene and other nitrogen metabolism genes under low- and normal-temperature treatment for 4 d. NT4d, normal-temperature treatment for 4 d. LT4d, low-temperature treatment for 4 d.

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