Genetic variations in ARE1 mediate grain yield by modulating nitrogen utilization in rice

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In crops, nitrogen directly determines productivity and biomass. However, the improvement of nitrogen utilization efficiency (NUE) is still a major challenge in modern agriculture. Here, we report the characterization of are1, a genetic suppressor of a rice fd-gogat mutant defective in nitrogen assimilation. ARE1 is a highly conserved gene, encoding a chloroplast-localized protein. Loss-of-function mutations in ARE1 cause delayed senescence and result in 10–20% grain yield increases, hence enhance NUE under nitrogen-limiting conditions. Analysis of a panel of 2155 rice varieties reveals that 18% indica and 48% aus accessions carry small insertions in the ARE1 promoter, which result in a reduction in ARE1 expression and an increase in grain yield under nitrogen-limiting conditions. We propose that ARE1 is a key mediator of NUE and represents a promising target for breeding high-yield cultivars under nitrogen-limiting condition.

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Nitrogen is an essential element for all living organisms and the nitrogen nutrients derived from plants are primary sources for humans and animals. In crops, nitrogen is one of the most predominantly limiting factors for productivity and breeding cultivars with the improved nitrogen utilization efficiency (NUE) is urgently demanding for sustainable development of agriculture. Most non-legume plants absorb inorganic nitrogen compounds from soil, followed by converting inorganic into organic nitrogen compounds, a process known as nitrogen assimilation. Plants mainly acquire nitrate (NO$_3^-$) and ammonium (NH$_4^+$) from soil by the plasma membrane-localized transporters. After entering the plant cell, nitrate is sequentially reduced to nitrite and ammonium by nitrate reductase and nitrite reductase, respectively, of which ammonium is converted into organic nitrogen compounds by the primary assimilation.

The biochemical framework of nitrogen assimilation has been well established, while the underpinning regulatory mechanisms are not well understood. The primary nitrogen assimilation is mediated by the coupled reactions catalyzed by glutamine synthetase (GS) and glutamate synthase, the latter one also known as glutamine:2-oxoglutarate amidotransferase (GOGAT). GS catalyzes the conversion of glutamate into glutamine by incorporating a molecule of ammonia, whereas GOGAT transfers an amide group from glutamine to 2-oxoglutarate to produce two molecules of glutamate. Two types of GOGAT, Fd-GOGAT and NADH-GOGAT, have been characterized, which use different reductants with distinctive tissue specificities and biochemical properties. The GS/GOGAT cycle is highly conserved in the plant kingdom, ranging from algae to higher plants. In rice (Oryza sativa L.), weak mutant alleles of fd-gogat (also known as abnormal cytokinin response1 or abcl) causes severe developmental defects and enhances bacterial blight resistance, whereas a double mutants were backcrossed with wild-type NPB plants three times, respectively, and the are1-1 and are1-2 single mutants were identified from respective BC$_2$F$_2$ progenies. These two alleles were phenotypically indistinguishable and are1-1 was used in most experiments hereafter unless indicated otherwise.

### Results

**Characterization of the are1 mutant.** We previously identified a rice abcl mutant (in the Nipponbare or NPB background) that shows a typical nitrogen deficient syndrome, including the reduction in the plant height, tiller number, chlorophyll level, and grain yield. $ABC1$ encodes Fd-GOGAT, a key enzyme in the GS/GOGAT cycle for nitrogen assimilation. We subsequently performed a genetic screen for abcl-1 repressor (are) mutants by examining the phenotype of the plant height, tiller number, and leaf color under field growth conditions. Two of those identified mutations, are1-1 and are1-2, partially rescued the pleiotropic phenotype of abcl-1, including defects in the plant height, tiller number and the leaf chlorophyll content (Fig. 1a–d and Supplementary Fig. 1a). In an allelism test, all F$_1$ progeny obtained from a cross between are1-1 and are1-2 showed an abcl-suppressor phenotype (Fig. 1a and Supplementary Fig. 1a), demonstrating that these two mutations are allelic. The abcl-1 are1-1 and abcl-1 are1-2 double mutants were backcrossed with wild-type NPB plants three times, respectively, and the are1-1 and are1-2 single mutants were identified from respective BC$_2$F$_2$ progenies. These two alleles were phenotypically indistinguishable and are1-1 was used in most experiments hereafter unless indicated otherwise.

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**Fig. 1** Identification and characterization of the are1 mutants. a Plants at the heading stage with the indicated genotypes. F$_1$ refers to F$_1$ plants obtained from crosses between abcl-1 are1-1 and abcl-1 are1-2. Scale bar, 15 cm. b–d Quantitative analysis of the plant height, tiller number, and soil-plant analysis development (SPAD, parameter indicating relative chlorophyll content) with the indicated genotypes at various growth stages. Data presented are mean values with s.d. (n = 30). e Wild-type (WT) and are1-1 plants at the indicated growth stages. Scale bar, 15 cm. f Flag leaves of WT and are1-1 plants 35 days post fertilization. Scale bar, 2 cm. g Quantitative analysis of SPAD in flag leaves of WT and are1-1 plants at the indicated growth stages. Data presented are mean values with s.d. (n = 40). h Analysis of Fd-GOGAT activity in leaves derived from 3-week-old seedlings with the indicated genotypes. Data presented are mean values of 4 technical repeats with s.d. **P < 0.01 (Student’s t-test)
During the early vegetative growth stages, are1 showed a phenotype similar to wild type. At the later growth stages, are1 had a slightly increased plant height and reduced tiller number (Fig. 1e). The heading date of are1-1 was often delayed for 3–5 days than wild type. At the grain-filling stage, are1-1 plants exhibited a stay-green phenotype (Fig. 1e, f). Consistent with this result, the accumulation of chlorophylls in are1-1 was significantly higher than that in wild type after the heading stage (Fig. 1g), indicating that are1-1 causes delayed senescence and prolonged efficient photosynthetic activities.

The abc1-1 is a leaky mutant allele, retaining approximately 50–60% Fd-GOGAT activity, and the T-DNA insertional mutant abc1-2 is a null allele10. Whereas the are1-1 mutation did not cause detectable effects on the accumulation of Fd-GOGAT protein (Supplementary Fig. 1b), the Fd-GOGAT enzymatic activity in abc1-1 are1-1 was nearly restored to that in the wild type (Fig. 1h). However, the abc1-2 are1-1 double mutant showed an abc1-2-like or seedling-lethal phenotype (Supplementary Fig. 1c), indicating that are1-1 is incapable of rescuing the phenotype of a null mutation in ABC1/Fd-GOGAT. These results suggest that the suppressing effect of are1 on abc1-1 is partly attributable to the rescued activity of Fd-GOGAT.

Enhanced tolerance of are1 to nitrogen deficiency. Given that the are1 mutations largely rescue the nitrogen deficient phenotype of abc1-1, ARE1 may be involved in nitrogen metabolism. Plants evolved various strategies for survival when grown under nutrient limiting environments, including the increase of the root-to-shoot ratio13. Compared to the wild type, are1-1 showed an increased root-to-shoot ratio under nitrogen deficiency condition, mainly resulted from increased root biomass (Fig. 2a and Supplementary Fig. 2a, b). Consistently, are1-1 seedlings retained a higher level of chlorophylls than the wild type (Fig. 2b and Supplementary Fig. 2c). Nitrogen depletion or reduction rapidly induced the expression of key genes involved in ammonia transport and nitrogen assimilation in wild-type rice seedlings, and this induction was reduced in are1-1 (Fig. 2c, d and Supplementary Fig. 2d–k), indicating that are1-1 is less sensitive to nitrogen starvation.

The reduced sensitivity of are1 to nitrogen starvation may be contributed by the increased accumulation of nitrogen in planta when grown under sufficient nitrogen condition. To test this possibility, we analyzed the nitrogen content in the field-grown plants. The total nitrogen contents in various organs were significantly higher in are1-1 than that in wild type plants, and the nitrogen uptake efficiency (NUpE) and NUE were consequently increased when the nitrogen fertilizer was progressively reduced (Fig. 2e, f and Supplementary Table 1). Consequently, the expression of the ammonia transport and nitrogen assimilation genes was increased in are1-1 than that in wild type plants under limited nitrogen supplies (Fig. 2g, h and Supplementary Fig. 2l–n), suggesting that ARE1 is negatively correlated to NUpE and NUE.

Molecular characterization of ARE1. In a genetic analysis, all F1 progeny (18 plants) obtained from a cross between are1-1 and NPB showed a wild-type-like phenotype by analyzing the senescence phenotype. In the F2 population, the normal and delayed senescence phenotypes were segregated approximately in a ratio of 3:1 (561:196 = 2.86:1; $\chi^2 \text{c} < \chi^2_{0.05} = 3.84$), indicating that are1 is a recessive Mendelian mutation in a single nuclear gene. Using a map-based cloning approach, we genetically mapped ARE1 to chromosome 8 in a region containing 13 predicted open reading frames (ORFs) (Fig. 3a; see Methods for details). DNA

![Fig. 2](image-url) Figure 2: Enhanced tolerance of are1-1 to nitrogen deficiency. a Analysis of the root/shoot ratio of 20-day-old wild-type (WT) and are1-1 plants grown in the absence or the presence of NH4NO3 (1.46 mM). Data presented are mean values with s.d. (n = 40). b Quantification of chlorophyll content in wild-type and are1-1 plants grown in the absence or the presence of NH4NO3 (1.46 mM) for the indicated times. Data presented are mean values with s.d. (n = 12). c, d Expression of OsAMT1;2 and OsNADH-GOGAT1 in response to nitrogen depletion. Two-week-old seedlings grown in a nitrogen-containing solution (see Methods) were transferred to a nitrogen-free solution (time 0), and then cultured for the indicated times. Total RNA was prepared from roots and used for qRT-PCR analysis. Data presented are mean values with s.d. (n = 12). e Analysis of nitrogen uptake efficiency (NUE) and nitrogen utilization efficiency (NUE) of 5-month-old WT and are1-1 plants grown under the indicated conditions. Data presented are mean values of 3 technical replicates with s.d. f, h Expression of OsAMT1;2 and OsNADH-GOGAT1 in 12-week-old WT and are1-1 plants grown under the indicated conditions. Data presented are mean values of 3 technical replicates with s.d. * * * $p < 0.05$ and $p < 0.01$ (Student’s t-test), respectively.
sequencing analysis revealed the presence of a single base deletion (A-899; the putative transcription start is referred to as +1) in the fourth exon of LOC_Os08g12780 in the are1-1 genome, resulting in frame-shift mutations after this base (Fig. 3a and Supplementary Fig. 3). In the are1-2 genome, a single base substitution (C-T) in the sixth exon caused a highly conserved Pro residue in frame-shift mutations after this base (Fig. 3a and Supplementary Fig. 3). ARE1 was predicted as a monocot homolog of the Arabidopsis ARE1 protein encoded by ARE1 (22.6% identity).

To verify the identity of the ARE1 candidate gene, we performed a molecular complementation test by transforming of abc1-1 are1-1 plants with a wild-type genomic fragment containing the entire LOC_Os08g12780 gene under the control of its native promoter (~2.3 kb) and 3′-untranscribed region (~1.9 kb). All the T1 transgenic plants showed an abc1-1-like phenotype (Fig. 3b), indicating that the transgene reverts the suppressing effect of are1-1 on the abc1-1 mutation. When transformed into are1-1 plants, the transgene rescued the delayed senescence phenotype of the mutant (Fig. 3c–e). These results demonstrate that LOC_Os08g12780 represents ARE1.

ARE1 encodes a putative protein that is highly conserved from lower to higher plants with unknown function (Supplementary Figs. 3 and 4a). Overall, ARE1 shares approximately 27–85% identity with ARE1-like proteins from cyanobacteria to flowering plants (Supplementary Fig. 4a). In the rice genome, a putative protein encoded by LOC_Os02g24598 shares low homology with ARE1 (22.6% identity). ARE1 is predominantly expressed in photosynthetic tissues, including leaves and leaf sheaths, with a lower expression level in culms, young panicles and spikes (Fig. 3f and Supplementary Fig. 4b–d). ARE1 was predicted as a chloroplast-localized protein containing a putative transit signal peptide of 47 amino acid residues (Supplementary Figs. 3 and 4e, f). When transiently expressed in rice protoplasts, ARE1-YFP (yellow fluorescent protein) fusion protein was localized in chloroplasts, whereas the deletion of the putative transit signal peptide retained the fusion protein in the cytoplasm (Fig. 3g).

Increased grain yield of are1 under low nitrogen conditions. Data presented above indicate that are1 is an important mediator of NUE. To assess the potential contribution of ARE1 to grain yield, we performed a multi-year field trial to analyze the major agronomic traits of are1-1 plants grown under various nitrogen conditions, in comparison with the wild-type NPB variety. The experiment was repeated for four years (2013–2016) and each sample included at least three duplicates. Under our assay condition, the saturated nitrogen fertilizer concentrations are approximately 280–300 kg/ha urea, similar to the routine usage by farmers in most rice-growing areas in China.

During early growth stages, no substantial difference was observed between NPB and are1-1 plants. However, after heading, are1-1 showed significantly delayed senescence than NPB under various nitrogen conditions (Fig. 4a and Supplementary Fig. 5a), accompanying with increased photosynthetic activities (Fig. 4b) and higher nitrogen utilization efficiency (Fig. 2c, f and Supplementary Table 1). Compared to that of NPB, although the plant height and the tiller number of are1-1 were marginally altered, the panicle length and the numbers of primary

Fig. 3 Molecular characterization of ARE1. a Genetic mapping of ARE1. The numbers (n) of recombinants used in mapping are given below the genetic maps (see Methods for details). Bottom, a schematic illustration of the ARE1 structure. Filled boxes and lines represent exons and introns, respectively. The mutation rates of are1-1 and are1-2 are shown below. b, c Molecular complementation of the are1-1 mutant phenotype. Plants at the grain-filling and dough stages with the indicated genotypes are shown. pARE1: abc1-1 are1-1 (b) and are1-1 (c) plants carrying a pARE1::ARE1 transgene, respectively. d Flag leaves derived from plants shown in (c). e Analysis of soil-plant analysis development (SPAD) in flag leaves of plants with the indicated genotypes at various growth stages. Data presented are mean values with s.d. (n = 40). f Analysis of the ARE1 expression in various organs by qRT-PCR. Data presented are mean values with s.d. (n = 3). g Analysis of subcellular localization of ARE1-YFP (yellow fluorescent protein) protein in rice protoplasts. Scale bars, 15 cm in (b, c), 2 cm in (d), and 5 μm in (g).
and secondary branches of panicles were increased in are1-1, resulting in an increase in grain number per panicle (Fig. 4c, d and Supplementary Fig. 5b–f). However, one-thousand-grain weight of are1-1 was slightly reduced than that of NPB (Fig. 4e). Overall, the grain yield of are1-1 plants increased by 10–20% than NPB grown under the nitrogen-limiting condition (40–60% of the saturated concentration; Fig. 4f and Supplementary Fig. 5g, h), under which conditions the differences of NUpE and NUE between NPB and are1-1 were also most apparent (Fig. 2e, f and Supplementary Table 1). Most notably, the grain yield of are1-1 grown under the nitrogen-limiting condition was comparable to that of NPB grown under the saturated urea condition (Fig. 4f and Supplementary Fig. 5g, h). These results indicate that are1-1 is a beneficial allele of high NUE and is particularly useful under nitrogen-limiting growth conditions.

Analysis of genetic variations in ARE1. Given that ARE1 plays an important role in mediating NUE and grain yield, we then explored potential association between these traits and genetic variations in ARE1. By analyzing a panel of Asian cultivated rice accessions from the 3000 Rice Genomes Project15,16, multiple variations were identified in the ARE1 promoters and coding sequences (Fig. 5a, b). In a panel of 2747 accessions, 15 single nucleotide polymorphisms (SNPs) were identified in the coding region, which were classified as 12 major haplotypes (Fig. 5b). Among these 15 SNPs, five variations caused substitutions in the coded amino acid residues, of which all occurred at very low frequencies (0.07–2.33%; Fig. 5b). The functional significance of these five SNPs remains elusive. In 2155 accessions with available data in the promoter region, three major haplotypes, ARE1NPB, ARE19311, and ARE1MH63, were identified, represented by the NPB, 9311 and Minghui63 (MH63) promoters, respectively. In these three haplotypes, three insertion–deletion polymorphisms (InDels) were identified in the promoter region of ARE1, of which the ARE19311 and ARE1MH63 haplotypes contained two small insertions of 6-bp at different positions (Fig. 5a).

Because of relatively more dramatic alterations in the genome structure, we focused on the analysis of the InDel polymorphisms in the ARE1 promoter. We found that the majority of japonica varieties were the ARE1NPB haplotype, with only 2.00, 3.50, and 4.46% occurrence of the ARE19311/ARE1MH63 alleles in the ancient japonica, temperate japonica, and tropical japonica accessions, respectively (Fig. 5c). However, the occurrence of the ARE19311/ARE1MH63 alleles was increased to 18.84% in the examined 1449 indica accessions, and higher frequency of the ARE19311/ARE1MH63 alleles was observed in aus (48.13%) and aromatic (100%) (Fig. 5c), suggesting that the ARE1 locus has been likely subjected to artificial selection during breeding.

Genetic variations modulate ARE1 expression and grain yield. To evaluate the biological function of these InDels, we examined the ARE1 expression levels in representative indica and japonica accessions of different haplotypes. All the analyzed ARE19311/ARE1MH63 haplotypes, regardless of their genetic backgrounds, had a decreased expression level of ARE1 than the ARE1NPB haplotypes under high nitrogen growth condition (Fig. 6a). Moreover, whereas low nitrogen treatment repressed the ARE1 expression, the ARE1NPB haplotypes were more sensitive to low nitrogen than the ARE19311/ARE1MH63 haplotypes (Fig. 6a). This result suggests that the ARE1 expression is negatively regulated by nitrogen availability and the genetic variations in the promoter of the ARE19311/ARE1MH63 accessions cause the reduced promoter activity and the responsiveness to nitrogen. Correlated to these results, the differences of grain yield between high and low nitrogen growth conditions in accessions carrying an ARE1NPB allele were generally greater than that of those carrying an ARE19311/ARE1MH63 allele (Fig. 6b and Supplementary Fig. 6a), suggesting that the two insertions in the ARE19311 and ARE1MH63 haplotypes are negatively correlated to both the ARE1 expression level and grain yield.

To further test whether these InDels are functional in a similar genetic background, we analyzed the phenotypes of a set of recombinant inbred lines (RILs) generated from crosses between two indica rice varieties 9311 and Peiai64 (PA64), also known as Liangyoupe9 (LYP9), a widely cultivated high-yield hybrid rice17. Of these two parents, PA64 was characterized as an ARE1NPB haplotype. In a panel of 134 RILs of LYP9, the ARE19311 InDel was tightly co-segregated with the phenotype of the reduced
ARE1 expression level (Fig. 6c), which, in turn, was negatively correlated to the grain yield under nitrogen-liminating conditions (Fig. 6d and Supplementary Fig. 6b).

In a transgenic study, overexpression of ARE1 caused the reduction in grain yield (Supplementary Fig. 6c, e, f). Conversely, the knockdown of the ARE1 expression by RNAi caused an increased grain yield under nitrogen-limited conditions (Supplementary Fig. 6d, g, h), indicating that the expression level of ARE1 is directly and negatively correlated to the grain yield.

Indica rice varieties generally have higher NUE than japonica varieties. We next asked if the are1-1 allele, which is in the japonica variety NPB genetic background, was also advantageous for high-yield indica varieties. In a near-isogenic line of MH63 carrying an are1-1 allele (MH63are1-1), the photosynthetic activity was substantially higher than its parent MH63, accompanying with an increase in the grain number and, eventually, an increase in grain yield (Fig. 6e and Supplementary Fig. 6i, j). Taken together, these results suggest that the genetic variations in the ARE1 promoter directly regulate its expression, thereby the rice productivity under nitrogen-liminating conditions.

Discussion

In this study, we have presented multiple lines of evidence demonstrating that ARE1 is a key determinant of the grain yield by modulating NUE in rice. Loss-of-function mutations in ARE1 largely rescue the nitrogen deficient syndrome caused by impaired nitrogen assimilation associated with the reduced Fd-GOGAT/ABC1 activity, indicating that ARE1 is a negative regulator of nitrogen assimilation. ARE1 is a chloroplast-localized protein with unknown function. Along with its subcellular localization, the enhanced photosynthetic activity and delayed-senescence phenotype of are1 mutants imply that ARE1 is not only involved in nitrogen assimilation, but may also be related to carbon metabolism. Consistent with this notion, the homologous gene of ARE1 in algae is implied in regulating photosynthesis by modulating CO2 transport. ARE1 shares approximately 27% homology with cotA protein of Synechocystis PCC6803 (see Supplementary Fig. 4a), which is also localized in chloroplasts. Mutations in the cotA gene caused reduced CO2 transport activity, presumably resulting from defects in light-induced proton extrusion. In Chlamydomonas reinhardtii, the chloroplast-localized Ycf10 protein, which shares homology with cotA, has been shown to promote inorganic carbon (CO2 and HCO3−) uptake into chloroplasts. Although the molecular mechanism of cotA-mediated and Ycf10-mediated inorganic carbon transport remains elusive, a similar biochemical activity may also be utilized by ARE1-like proteins in higher plants to modulate photosynthesis, which provide the carbon skeletons, energy and reductants for nitrogen metabolism. Understanding the precise biochemical function of ARE1 will be crucial to reveal the mechanism of ARE1-mediated carbon and nitrogen metabolism. Because the GS/Fd-GOGAT cycle is a key converging point of C/N balance that is directly linked to the TCA cycle, a key question to be addressed is the biochemical and molecular mechanisms of the repressing effect of ARE1 on the Fd-GOGAT activity.

Genetically, a suppressor may act upstream or downstream of the modified locus. Because are1-1 partially rescued the phenotype of the weak allele abc1-1, but not that of the null allele...
abc1-2, the ARE1 function should be dependent on ABC1. Therefore, ARE1 may genetically act upstream of Fd-GOGAT/ABC1. In agreement with this notion, whereas gogat1, which carries a substitution mutation in Fd-GOGAT/ABC1, shows an early senescence phenotype and an increase in grain nitrogen deposition, \( \text{are}1-1 \) shows an opposite phenotype, further suggesting that ABC1 and ARE1 act in a linear genetic pathway. Moreover, we notice that the ARE1 expression level is substantially increased in the rice grain number, plant height, and heading date \( \text{ghd7} \) mutant, indicating that the expression of ARE1 is negatively regulated by Ghd7. As implied from its name, Ghd7 regulates multiple traits, including the grain number and chlorophyll contents, which are also affected by the \( \text{are}1 \) mutations. We speculate that Ghd7 may act genetically upstream to regulate the ARE1 expression, thereby modulating a subset of similar physiological and developmental processes.

The structure of the ARE1 promoter shows significant variations among a large pool of rice varieties. The two small insertions in the ARE1 promoter are rarely found in all \( \text{japonica} \) varieties, but the occurrence is significantly increased in \( \text{indica} \) and \( \text{aus} \) accessions, implying that these genetic variations may have been artificially selected during breeding. We speculate that these two InDels were selected in some \( \text{japonica} \) accessions, implying that these genetic variations may have been artificially selected during breeding. We speculate that these two InDels were selected in some \( \text{japonica} \) accessions, implying that these genetic variations may have been artificially selected during breeding. We speculate that these two InDels were selected in some \( \text{japonica} \) accessions, implying that these genetic variations may have been artificially selected during breeding. We speculate that these two InDels were selected in some \( \text{japonica} \) accessions, implying that these genetic variations may have been artificially selected during breeding. We speculate that these two InDels were selected in some \( \text{japonica} \) accessions, implying that these genetic variations may have been artificially selected during breeding.

Finally, when grown under nitrogen-limiting conditions, the grain yield of both \( \text{japonica} \) and \( \text{indica} \) varieties carrying an \( \text{are}1-1 \) allele is comparable to that of their parents supplied with the saturated amount of nitrogen fertilizers. Therefore, discoveries...
made in this study present a potential solution of reducing the application of nitrogen fertilizers in rice production. Because of the conservation of ARE1-like genes, a similar approach may also be applicable to other crops.

Methods

Plant materials and growth conditions. The are1-1 mutant is a weak mutant allele carrying a single substitution mutation in Fd-GOGAT/ABC1 in the Nipponbare background, and the are1-2 mutant is a T-DNA insertion mutant (PFG_3A-01082) in the Dongjin background. The are1-1 seeds were mutagenized by ethyl methane sulfonate and M_{2} seeds (approximately 28800 seeds) obtained from self-pollinated M_{1} plants were used for screening of putative suppressors under field growth conditions. Screening was performed by manually analyzing the plant height, tiller numbers and leaf color during the primary round of screen. Putative are1 mutants were subjected to the secondary round of screen in the M_{3} generation and then backcrossed with wild-type (Nipponbare; NPB) or are1-1 mutant plants for at least three times. The M_{3}H63embryos were constructed by backcrossing of are1-1 with MH63 for seven times, and resulting BC_{2}F_{2} population were used in this study. The LYP9 recombinant inbred lines (RILs) were generated from a cross between an indica variety 9311 and a maternal indica variety PAC4, a photo-thermosensitive male sterile line. The inbred F_{1} generation was used in this study.

Plant growth. Planted fields in Beijing, Jinan (Shandong province), Hangzhou (Zhejiang province), and Lingshui (Hainan province) with routine management. For hydroponic grown seedlings, seeds were surface-sterilized with 75% ethanol for 5 min and then sterilized with 30% bleach for 30 min, followed by washing with sterile water for six times. Sterilized seeds were germinated in distilled water for 36–48 h at 37 °C in darkness, and then transferred to a modified hydroponic culture solution and fresh solution was changed every 3 days. The modified hydroponic culture solution contains variable concentrations of NH_{4}NO_{3} (0, 0.15, 0.75, or 1.46 mM), 0.37 mM CaCl_{2}, 0.53 mM MgSO_{4}, 0.18 mM KH_{2}PO_{4}, 0.09 mM K_{2}SO_{4}, 46.26 μM H_{2}BO_{3}, 0.32 μM CuSO_{4}, 0.77 μM ZnSO_{4}, 9.15 mM MnSO_{4}, 0.84 mM MoO\textsubscript{3}, 5.37 μM FeEDTA, and 0.16 mM Na_{2}EDTA. When transferring between hydroponic culture solutions containing different concentrations of NH_{4}NO_{3}, seedlings were washed in distill water for 3–5 times. Rice seedlings were cultured in a greenhouse or a growth chamber with 70% relative humidity under 12 h/12 h light/dark and 25–30 °C/25 °C day/night conditions.

Genetic transformation of rice was performed as described. Briefly, rice embryonic calli were infected with agrobacteria cultures for 1–2 min, followed by extensive washing with sterilizer. The infected calli were cultured on N6-AS medium (3.981 g/L N6 salts, 2 mg/L 2,4-D, 0.5 g/L casein hydrolysate, 0.5 g/L sucrose, 10 g/L glucose, 15 mg/L acetylsyringone, 4 g/L phytagel, pH 5.2) for 3 days at 25 °C and then washed with sterilizer water containing 500 mg/L carbencillin for three times. The calli were cultured on selection medium (3.981 g/L N6 salts, 2 mg/L 2,4-D, 0.5 g/L casein hydrolysate, 0.5 g/L L-proline, 30 g/L sucrose, 4 g/L phytagel, 200 mg/L carbencillin, 200 mg/L cefotaxime, 50 mg/L hygromycin B, pH 5.8) for 4 weeks to selecting the ARE1::GUS transgenic embryos. The positive transgenic embryos were then transferred to differentiation medium (4.4 g/L MS salts, 30 g/L sucrose, 0.5 g/L sorbitol, 2 g/L casein hydrolysate, 0.02 mg/L NAA, 2 mg/L kinetin, 4 g/L phytagel, 50 mg/L carbencillin, 50 mg/L hygromycin B, pH 5.8) and cultured for 4–8 weeks. The regenerated plantlets were cultured on rooting medium (2.22 g/L MS salts, 30 g/L sucrose, 30 g/L sucrose, 0.5 mg/L carbenicillin, 0.5 g/L phytagel, pH 5.8) for several weeks and then grown in soil in a greenhouse under rooting conditions.

Field trials. A randomized block design approach was used to analyze the major agronomic traits under various nitrogen conditions. Rice plants were cultivated with a distance of 15 × 25 cm in a 3.8–4.0 m plot and each sample or treatment included six replicates (plots). Urea was used as the only nitrogen source and applied for three times at the seedling, tillering and booting stages, with 30, 40, and 30% of total applied urea at each stage, respectively. Potassium sulfate (120 kg/ha) and calcium phosphate (260 kg/ha) were used as phosphorus and potassium fertilizers, supplied when rice seedling transplanting.

Plasmid construction. Plasmids were constructed following standard methods as described. To construct pARE1::ARE1, three partially overlapped DNA fragments (5000, 2000, and 3227 bp, respectively) were PCR-amplified using primer ABE629-352F and BAMS1099-31-PU; 1:10000 dilution) were used as primary antibodies and HRP-conjugated goat anti-rabbit IgG or HRP-conjugated goat anti-mouse IgG (Dingguo Changsheng Biotech, Beijing, China; 1:50000 dilution) were used as secondary antibodies. The signal was detected using a SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Scientific, Cat.: 34096) according to the manufacturer’s instructions.

Transient expression assays in rice protoplasts. Rice protoplasts were prepared as described with minor modification. Briefly, stem and sheath tissues derived from 14-day-old rice seedlings were cut into 0.5–1.0 mm stripes. The sample was

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immediately transferred into 150 mL of 0.6 M mannitol and incubated for 10 min, followed by incubation in an enzyme solution (0.6 mL mannitol, 10 mM MES, pH 5.7, 10 mM CaCl₂, 5 mM β-mercaptoethanol, 1% [v/v] cell suspension, 0.75% maceratine R-10, 0.1% BSA, and 50 μg/mL carbeneillin) for 4–5 h at 28 °C with gentle shaking (40–60 rpm) in darkness. Protoplasts were collected using a 35 μm mesh filter and resuspended in one volume of W5 solution (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, and 2 mM MES, pH 5.7) by inverting for 5–8 times, followed by centrifugation at 150×g for 3 min. Protoplasts were then resuspended in MMC solution (0.6 M mannitol, 15 mM MgCl₂, and 4 mM MES, pH 5.7) at a concentration of 1–5 × 10⁶ cells/mL. The viability of protoplasts was determined by the FDA staining method as described40.

For transient expression assays, 5 μg plasmid DNA were introduced into 50 μL freshly prepared protoplasts by PEG-mediated transformation as described41. Briefly, 55 μL freshly prepared PEG solution (0.6 M mannitol, 100 mM CaCl₂ and 40% PEG4000) was gently added, and then incubated for 15 min at 28 °C in darkness, followed by gently mixed with ten volumes of W5 solution and inverted for 5–8 times. The protoplasts were pelleted by centrifugation at 150g for 3 min, and then gently resuspended in W5 solution.

For the analysis of subcellular localization, after transformation with appropriate expression vectors, protoplasts were cultured for 12–16 h in darkness at 28 °C, and then fluorescence signals were visualized and scanned under a confocal laser scanning microscope (Leica TCS SPS). At least 50 cells were analyzed in each sample.

**Analysis of Fd-GOGAT enzyme activity.** Analysis of Fd-GOGAT enzyme activity was performed as described42,43 with minor modifications. Briefly, plant extracts were prepared by grinding 2 g fresh leaves of 3-week-old seedlings in 1 mL of cold extraction buffer (50 mM HEPES, pH 7.5, 15 mM KCl, 1 mM EDTA, 1 mM DTT, and 0.1% BSA, and 50 μL reaction mixture [50 mM HEPES, pH 8.5, 1% (v/v) glycerol, 1 mM NADPH]). Each reaction mixture was added to 200 μL reaction buffer (50 mM HEPES, pH 8.5, 1% (v/v) glycerol, 1 mM NADPH), and corrected by the rate measured from the reaction mixture with no enzyme. The Fd-GOGAT activity (nmoles NADPH oxidized (37 °C, pH 8.5)/min/mg protein) was measured spectrophotometrically by recording the rate of NADPH oxidation at 340 nm (indicated by a change in A340 nm) using a Beckman Coulter, US) for the Kinetics/Time Run. The Fd-GOGAT activity [nmoles NADPH oxidized (37 °C, pH 8.5)/min/mg protein] was measured spectrophotometrically by recording the rate of NADPH oxidation at 340 nm (indicated by a change in A340 nm) using a Beckman Coulter, US) for the Kinetics/Time Run.

**Determination of total chlorophyll content and SPAD efficiency.** Total chlorophyll content and SPAD efficiency (NUpE) was determined by the ratio of total chlorophyll concentration to the absorbance of the supernatant at 649 and 665 nm as described44. To measure total chlorophyll content and SPAD efficiency, plants were homogenized in a MM 400 grinding apparatus (Retsch, Germany), followed by centrifugation at 2000×g for 10 min. The supernatant was recovered, and the absorbance at 649 and 665 nm was measured using a spectrophotometer (PerkinElmer, USA) to determine the total chlorophyll content.

**Data availability.** The authors declare that all data supporting the findings of this study are available within the manuscript or its supplementary files or are available from the corresponding authors upon request.

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Author contributions
J.Zuo., J.L., Q.Q., Q.W., X.X., and F.C. designed the experiments and analyzed the data. Q.W., J.N., and X.X. performed most experiments, with assistance of J.Zhang, J.B., G.D., J.H., B.B., L.C., Q.X., J.F., X.Y., and J.P. H.Y. and Q.W. performed the genome sequence analyses. J.Zuo. and Q.W. wrote the paper. All authors read and commented on the paper.

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