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GENES ASSOCIATED WITH NITROGEN METABOLISM IN RICE

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ABSTRACT
Nitrogen metabolism is a primary process in crop. Rice, a monocotyledonus crop is for its favourable anaerobic root zone condition can uptake both nitrate and ammonium forms of nitrogen sources for subsequence transport and assimilation in translocation system of rice plant. Thus, nitrogen utilization efficiency (NUE) is important for nitrogen economy and environmental safety. An attempt has been undertaken to describe the genes taking part in nitrogen uptake, transport, assimilation in nitrogen metabolic pathways in rice and their roles and interactions amongst themselves. Informations are also made available of the mutants and their performances and mode of actions briefly. Some transcriptomics and DEGs are mentioned in normal, nitrogen stress conditions to know the efficient rice plants can be grown under low nitrogen with high grain yield and NUE. Possible changes of nitrogen metabolic genes (uptake, transport, assimilation and remobilization genes) in abiotic stresses are elaborated.

Keywords: *Rice, Nitrogen, Uptake, Transport, Metabolism, Genes, Mutants*

INTRODUCTION
Nitrogen is an essential nutrient for plants and an important factor limiting crop productivity. Applying nitrogen fertilizer to paddy fields is an important way of improving rice yields. However, applying excess nitrogen fertilizer decreases both the nitrogen absorption efficiency and the efficiency of the fertilizer, and contributes to pollution of the environment with nitrogen. Fully exploiting the genetic potential of rice to make use of nitrogen could allow nitrogen absorption and use by rice to be effectively improved. Nitrogen metabolism by rice is a complex dynamic process involving numerous physiological and biochemical processes, including nitrogen transport, distribution, use, and reuse (Ladha *et al.,* 1998). Nitrogen uptake and use by rice are not independent, but rather form a cyclic metabolic system mediated by a series of enzymes. NR, glutamine synthetase (GS), glutamic acid synthase (GOGAT), and glutamate dehydrogenase are the key enzymes involved in nitrogen metabolism by plants (Marschner, 2012).
Rice is mostly cultivated in anaerobic paddy field soils, where ammonium (NH_4^+) rather than NO_3^- predominates as N source. Nevertheless, specialized aerenchyma rice root cells transfer oxygen from shoot to root, causing release into the rhizosphere. The resultant enhancement of soil bacterial conversion of NH_4^+ to NO_3^- (nitrification) (Li *et al.,* 2008) provides 15–40% of paddy field grown rice N uptake (Yang *et al.,* 2016).

Nitrate Uptake,Transport and Assimilation
The *indica* and *japonica* rice (*Oryza sativa*) subspecies differ in nitrate (NO_3^-) assimilation capacity and nitrogen (N) use efficiency (NUE). It was revealed that a major component of this difference is conferred by allelic variation at OsNR2, a gene encoding a NADH/NADPH-dependent nitrate reductase (NR). Selection-driven allelic divergence has resulted in variant *indica* and *japonica* OsNR2 alleles encoding structurally distinct OsNR2 proteins, with *indica* OsNR2 exhibiting greater NR activity. *Indica* OsNR2 also promotes NO_3^- uptake via feed-forward interaction with OsNRT1.1B, a gene encoding a NO_3^- uptake transporter. These properties enable *indica* OsNR2 to confer increased effective tiller number, grain yield and NUE on *japonica* rice, effects enhanced by interaction with an additionally introgressed *indica* OsNRT1.1B allele. In consequence, *indica* OsNR2 provides an important breeding resource for the sustainable increases in *japonica* rice yields necessary for future global food security (Fig.1).

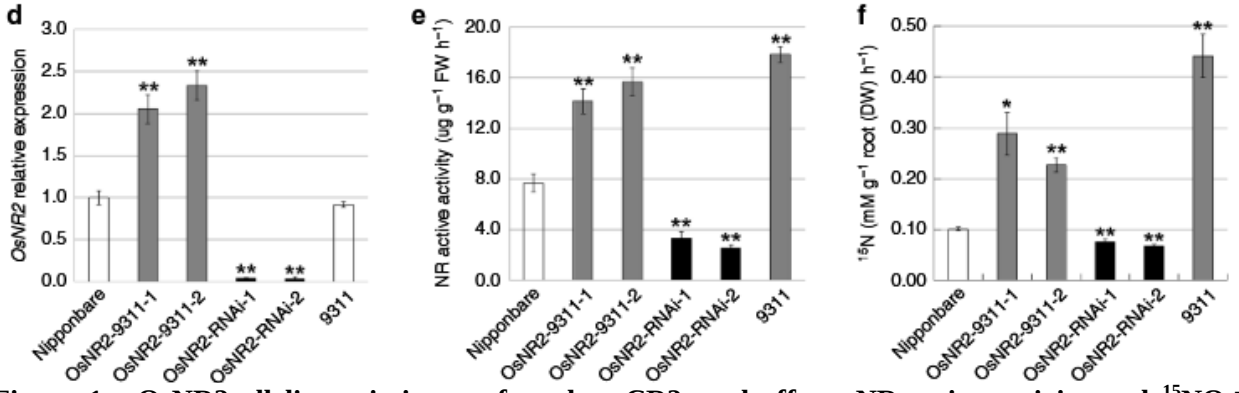

Figure 1: OsNR2 allelic variation confers the qCR2, and affects NR active activity and $^{15}NO_3^-$ uptake. d Leaf OsNR2 mRNA abundance, e leaf NR active activity, f $^{15}NO_3^-$ uptake activity of roots exposed to 1.25mM $^{15}NO_3^-$. Value is mean ± s.d. (n =3 for d–f) (After Gao *et al.*, 2019)

A recent report (Bao *et al.*, 2015) suggested that, in addition to functioning in NH_4^+ uptake in the roots, *OsAMT1.3* may act as a signal sensor to regulate plant growth, carbon, and nitrogen use efficiency (NUE). Similarly, *OsAMT1.3* has been suggested as being involved in the adaptation ability of rice to low NH_4^+ supplies (Ferreira *et al.*, 2015). In the same way, *OsAMT2.3* was reported to be affected by N supply (Gaur *et al.*, 2012).N transport in the form of NH_4^+ is facilitated by a group of well-identified NH_4^+ transporters, including *OsAMT1.3* and *OsAMT2.3* genes.

Investigations of the transcriptional regulation of major genes involved in the NUE in rice treated with $KClO_3$, which acts as an inhibitor of the reducing activity of nitrate reductase (NR) in higher plants with a set of two $KClO_3$ sensitive nitrate reductase (NR) and two nitrate transporter (NRT) introgression rice lines (BC2F7), carrying the *indica* alleles of NR or NRT, derived from a cross between Saeilmi (*japonica*, P1) and Milyang23 (*indica*, P2), were exposed to $KClO_3$ at the seedling stage. The phenotypic responses were recorded 7 days after treatment, and samples for gene expression, physiological, and biochemical analyses were collected at 0 h (control) and 3 h after $KClO_3$ application. The results revealed that Saeilmi (P1, *japonica*) and Milyang23 (P2, *indica*) showed distinctive phenotypic responses. In addition, the expression of *OsNR2* was differentially regulated between the roots, stem, and leaf tissues, and between introgression lines. When expressed in the roots, *OsNR2* was downregulated in all introgression lines. However, in the stem and leaves, *OsNR2* was upregulated in the NR introgression lines, but downregulation in the NRT introgression lines. In the same way, the expression patterns of *OsNIA1* and *OsNIA2* in the roots, stem, and leaves indicated a differential transcriptional regulation by $KClO_3$, with *OsNIA2* prevailing over *OsNIA1* in the roots. Under the same conditions, the activity of NR was inhibited in the roots and differentially regulated in the stem and leaf tissues. Furthermore, the transcriptional divergence of *OsAMT1.3* and *OsAMT2.3*, *OsGLU1* and *OsGLU2*, between NR and NRT, coupled with the NR activity pattern in the roots, would indicate the prevalence of nitrate (NO_3^-) transport over ammonium (NH_4^+) transport.

It shows that OsNia3 is a member of the rice nitrate reductase (NR) gene family.OsNia3 was found to be expressed in stem nodes of rice, while the loss of its function led to a reduction in plant height mainly due to disrupted nitrate metabolism at the site of OsNia3 expression. The expression levels of genes related to nitrate transport, nitrate assimilation, and ammonium assimilation, such as OsNia1, OsNRT2.3A, and OsNGS1, increased in the leaves of osnia3 mutant plants, concomitant with an increase in the activity of the nitrogen metabolism pathway. The ammonium and amino acid contents in the leaves also increased in the osnia3 mutant, as did the chlorophyll content. Phenotypic analysis showed that the osnia3 mutant exhibited a shorter growth period compared with wild-type plants; nevertheless, yield was not significantly reduced (Kabange *et al.*, 2021).

In recent years, CRISPR/Cas9 technology was employed to uncover the function of genes involved in the N metabolism pathway. It was found that flowering in the osnia3 mutant with deletion of the NIA like (LOC4345798, OsNia3), occurred 10-15 days earlier than that of the wild type (WT) (Nanchang, Jiangxi), without significantly affecting yield.

Nitrogen assimilation is a complex biochemical process catalyzed by the coupled reactions of glutamine synthetase (GS; EC6.3.1.2) and glutamate synthase (Xu *et al.,* 2012). GS catalyzes the conversion of glutamate into glutamine by incorporating a molecule of ammonia. Two types of GS, cytosolic GS1 and chloroplast GS2, were characterized by biochemical studies (Lam *et al.,* 1996). GS1 is presumably involved in the generation of glutamine for primary nitrogen assimilation in roots (Miflin, 1974) and for intercellular nitrogen transport in vascular bundles (Carvalho *et al.,* 1992; Kamachi *et al.,* 1992), whereas GS2 functions in the reassimilation of photorespiratory ammonia in leaves (Kendall *et al.,* 1986).

Glutamate synthase, also known as glutamine 2-oxoglutarate amidotransferase (GOGAT), catalyzes the transfer of an amide group from glutamine to 2-oxoglutarate (2-OG) to produce two molecules of glutamate. In plants, two types of GOGAT, ferredoxin-dependent (Fd)-GOGAT (EC 1.4.1.7), and NADH-GOGAT (EC 1.4.1.14), were characterized, which use different electron donors in the amido transfer reactions (Forde and Lea, 2007). Although both types of GOGAT are localized in the plastid, they show distinctive tissue specificities and biochemical properties. Whereas Fd-GOGAT has the highest activity in photosynthetic tissues by directly utilizing light energy as a supply of the reductant, NADH-GOGAT is predominantly present in non-photosynthetic tissues utilizing the reductant supplied by the pentose phosphate pathway (Forde and Lea, 2007). The high level of Fd-GOGAT in leaves correlates with its critical role in nitrogen reassimilation in photosynthetic tissues. On the other hand, NADHGOGAT is proposed to function in primary nitrogen assimilation and reassimilation of ammonia released during amino acid catabolism (Lam *et al.,* 1996; Suzuki and Knaff, 2005). In the model monocotyledonous plant rice (Oryza sativa L.), Fd-GOGAT represents the major activity of GOGAT and has been biochemically characterized (Yamaya *et al.,* 1992). The rice Fd-GOGAT is highly abundant in mesophyll cells and other chloroplast-containing cells (Ishiyama *et al.,* 1998). Whereas the rice Fd-GOGAT gene has been partially characterized (Mattana *et al.,* 2006). The coupled GS/GOGAT reactions act as a linker that directly connects nitrogen metabolism and carbon metabolism together (Hodges, 2002).

The absorption and utilization of NO_3 in rice is a complex process, including absorption, translocation and assimilation. In order to adapt to the changing environment, plants have evolved two nitrate transport systems, low affinity transport systems (LATS)and high affinity transport systems (HATS), which are responsible for NRT1/PTR (NPF) family members and NRT2 family members respectively. The NRT2 family in rice includes OsNRT2.1, OsNRT2.2, OsNRT2.3a, OsNRT2.3b, and OsNRT2.4 (Wang *et al.,* 2018; Song *et al.,* 2020; Wei *et al.,* 2018). OsNRT2.1, OsNRT2.2, and OsNRT2.3a need to interact with OsNAR2.1 protein to have NO_3 transport activity. OsNRT2.3a was involved in the transport of NO_3 from root to shoot.

A subsequent study found that OsNRT2.3b did not need the interaction of OsNAR2.1 and could transport NO_3 alone. Overexpression of OsNRT2.3b increased the buffering capacity of cell pH and significantly improved the grain yield and NUE of rice (Fig.2). Previous results showed that the expression of pOsNAR2.1 OsNAR2.1 could increase the NO_3 uptake rate at seedling stage, significantly promote rice growth, and improve grain yield and agronomic NUE. The expression of pOsNAR2.1 OsNRT2.1 increased the expression ratio of OsNRT2.1 to OsNAR2.1 in stem, and increased grain yield and NUE in rice. Co-overexpression of OsNAR2.1 and OsNRT2.3a can improve the biomass and nitrate transport efficiency, and ultimately improve the grain yield and NUE in rice (Chen *et al.,* 2020) (Table1). A putative nitrate transporter gene OsNPF4.5 was induced by mycorrhizal, which is exclusively expressed in the cells containing arbuscules and displayed a low-affinity NO_3 transport activity involved in symbiotic N uptake (Wang *et al.,* 2020).

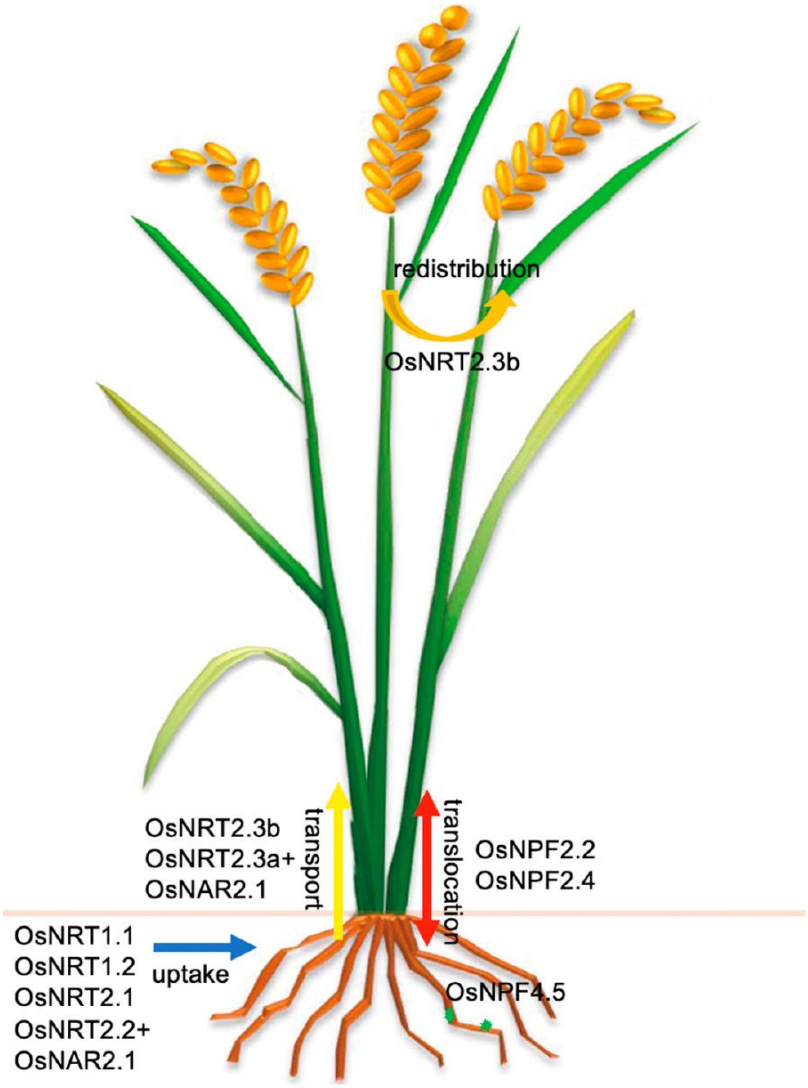

Figure 2: Summary of the contribution of NO_3^- transporters in rice. OsNRT1.1, OsNRT1.2, OsNRT2.1, and OsNRT2.2 (cooperating with OsNAR2.1) are responsible for NO_3^- uptake from the soil. OsNRT2.3a cooperating with OsNAR2.1 is responsible for root-to-shoot NO_3^- transport, and OsNRT2.3b is also responsible for transporting NO_3^- to the shoot and remobilizing N into the grain. OsNPF2.2 and OsNPF2.4 are involved in NO_3^- translocation. OsNPF4.5 is induced by mycorrhizal, involved in symbiotic N uptake. Blue line means NO_3^- uptake, yellow line means transport NO_3^- from root to shoot, red line means NO_3^- translocation, orange line means NO_3^- redistribution.

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 NH_4^+-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 (Fig.3) 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. Phosphorylation of NR is a steady-state regulatory mechanism of nitrogen metabolism, and dephosphorylation of NIA1 protein improved NR activity and nitrogen utilization efficiency. Excessive

Table 1: Candidate NO_3 transporter and regulation genes for crop (rice) application, NUE, nitrogen use efficiency

Gene	Acc.No.		Chacteristics	Nitrogen behavior	Reference
OsTCP19	Os06g12230		Responding to nitrate	Negatively regulate tillering. High tillering response to N	Liu *et al.*, 2021
OsNRT2.3a	AK109776		Root-shoot NO_3 transport	Transport	Tang *et al.*, 2012
OsNRT2.3b	AK072215		Enhancing p^H homeostasis,grain yield and NUE	Uptake	Fan *et al.*, 2016
OsNAR2.1	NC_029257.1		Interaction withOsNRT2.1/2.2/2.3a	Uptake and transport	Yan *et al.*, 2011; Liu *et al.*, 2014
OsNRT1.1B	Os10g40600		Improving NUE in indica rice	Uptake and transport	Hu *et al.*, 2015
OsNPF2.2	Os12g44100		Unloading NO_3 from xylem and root to shoot transport	Transport	Li *et al.*, 2015
OsNPF2.4	AK099321.1		Long-distance NO_3 transport and regulating NO_3/K^+ shuttle	Uptake and Transport	Xia *et al.*, 2015
OsNRT2.4	Os01g36720		Dual affinity nitrate transporter and regulate root growth	Uptake	Wei *et al.*, 2018
OsNRT1.1A	Os08g05910		Regulating N utilization and flowering	Uptake	Wang *et al.*, 2018
OsNPF4.5	Os01g54515		Low affinity nitrate transport involving symbiotic N uptake	Uptake	Wang *et al.*, 2020
OsNPF6.1	Os01g01360		OsNPF6.1HapB enhances nitrate uptake and confers high NUE by increasing yield under low N supply.	Uptake	Wang *et al.*, 2018
OsNPF7.1	Os07g41250		Regulates tillering and grain yield	Uptake	Huang *et al.*, 2019
OsNPF7.4	Os04g50940		"	Uptake and transport	Huang *et al.*, 2019
OsNPF7.2	Os02g47090		Positively regulates tillering and grain yield	"	Wang *et al.*, 2018
OsNPF7.7	Os10g42870		Regulates shoot branching and NUE	uptake	Huang *et al.*, 2018
OsNPF7.3	Os04g50950		N allocation and grain yield	Translocation	Fang *et al.*, 2017
OsPTR9	Os06g49250		NUE and grain yield	uptake	Fang *et al.*, 2013

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. In rice, the overexpression of OsAAP1 resulted in increased N uptake and redistribution, as well as significantly increased tiller number and final yield (Ji *et al.*, 2020). OsAAP3 and OsAAP5 are mainly expressed in the vascular system of rice, and may participate in the transport of amino acids between xylem and phloem (Lu *et al.*, 2018; Wang *et al.*, 2019).

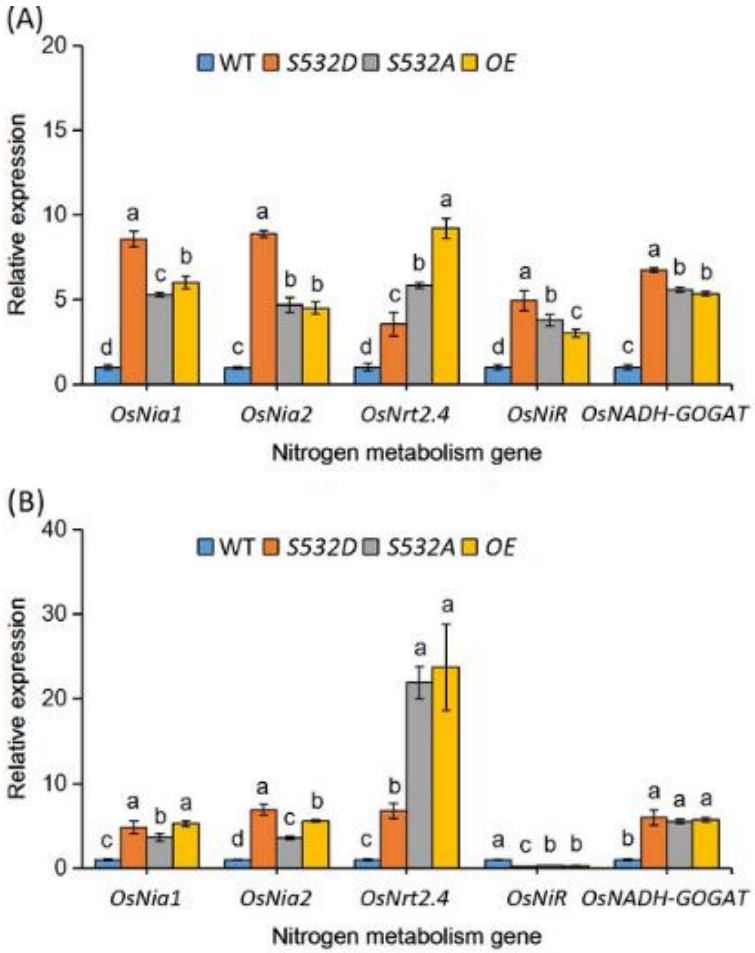

Figure 3: Analysis of gene expression related to nitrogen metabolism. (A) Expression analysis of nitrogen metabolism-related genes at normal temperature for 4 d. (B) Expression analysis of nitrogen metabolism-related genes at low temperature for 4 d. Column data are means, and short lines represent mean 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.

Genetic association analyses with RNAi lines of OsAAP3 and OsAAP5 also showed significant improvements in tiller number and 68 representatives *Japonica* or *Indica* germplasms identified that OsLHT1 has a natural variation in aspartate absorption between *Japonica* and *Indica*, and OsLHT1 functions in a broad spectrum of amino acids, and effectively transported aspartate, asparagine, and glutamate in yeast cells. OsLHT1 is responsible for both the root uptake and root to shoot allocation of a broad spectrum of amino acids in rice (Guo *et al.*, 2020).

When *Indica* OsNR2 and OsNRT1.1b were concurrently expressed, indicating that OsNR2 could regulate OsNRT1.1b and thus nitrate uptake (Gao *et al.*, 2019). The overexpression of OsNADH-GOGAT in rice by its native promoter also resulted in increased grain weight at low N fertilization (Yamaya *et al.*, 2002).

Interestingly, the combined overexpression of OsAMT1;2 and OsNADH-GOGAT1 in rice can increase NUE in rice under both sufficient and low nitrogen conditions (Taochy *et al.*, 2015).
Nitrogen availability affected the internal nitrogen compounds of rice leaves, such as the concentrations of total protein and total free amino acids. Consistently, the integrated analysis of transcriptome and metabolome showed that, compared with normal nitrogen, nitrogen transport, assimilation, and amino acid metabolism changed significantly under low and high nitrogen. Ammonium transporter gene OsAMT1.2 and nitrate transporter gene OsNRT2.5 were upregulated under low N, while nitrate transporter gene OsNRT2.5 was downregulated under high N.
The gene encoding nitrate reductase (OsNIA1) was induced by low N but inhibited by high N. The gene encoding NADH-dependent glutamate synthase (OsGLT1) was down regulated under low N but upregulated under high N. The metabolome profile showed that glutamine and glutamate were decreased under low N and increased under high N.

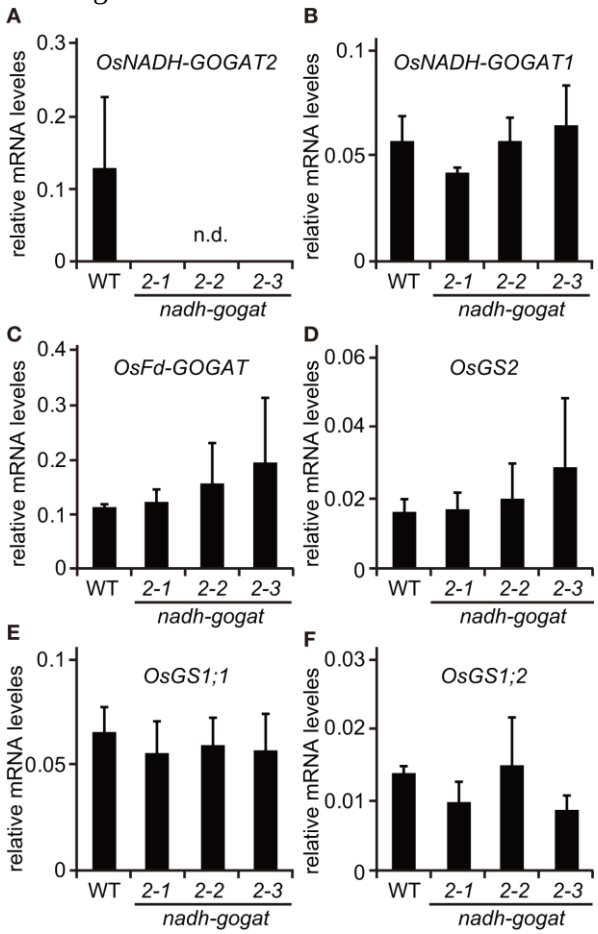

Figure 4: Real-time PCR detection of mRNAs for OsNADH-GOGAT 2 (A), OsNADH-GOGAT 1 (B), OsFd-GOGAT (C), OsGS2 (D), OsGS1;1 (E), and OsGS1;2 (F) from a flag leaf of wild-type (WT) and Tos17-inserted homozygous lines grown with soil in a green house. Three knockout lines, ND2034, NC0418, and NG3002, were indicated as in nadh-gogat2-1, 2-2, and 2-3 respectively. mRNA contents were normalized using actin mRNA. Means of independent triplicate samples and SD are indicated (After Tamura *et al.*, 2011).

Physiological role of NADH-GOGAT2 is not yet known. It reveals that isolated retrotransposon mediated-knockout mutants lacking OsNADH-GOGAT 2. In rice grown under paddy field conditions, disruption of the OsNADH-GOGAT 2 gene caused a remarkable decrease in spikelet number per panicle associated with a reductions in yield and whole plant biomass, when compared with wild-type (WT)

plants. The total nitrogen contents in the senescing leaf blade of the mutants were approximately a half of the WT plants. Expression of this gene was mainly detected in phloem companion cells and phloem parenchyma cells associated with large vascular bundles in fully expanded leaf blades, when the promoter region fused with a β-glucuronidase gene was introduced into the WT rice (Fig.4). These results suggest that the NADH-GOGAT2 is important in the process of glutamine generation in senescing leaves for the remobilization of leaf nitrogen through phloem to the panicle during natural senescence. These results also indicate that other GOGATs, i.e., NADH-GOGAT1 and ferredoxin-GOGAT are not able to compensate the function of NADH-GOGAT2.

Nitrogen Stress

Mining low-nitrogen tolerant rice genes and improving nitrogen use efficiency are of great significance to the sustainable development of agriculture. This study was conducted by Genome-wide association study on a basis of two roots morphological
traits (root length and root diameter) and 788,396 SNPs of a natural population of 295 rice varieties. The transcriptome of low-nitrogen tolerant variety (Longjing 31) and low-nitrogen sensitive variety (Songjing 10) were sequenced between low and high nitrogen treatments. A total of 35 QTLs containing 493 genes were mapped. 3085 differential expressed genes were identified. Among these 493 genes, 174 genes showed different haplotype patterns. There were significant phenotype differences among different haplotypes of 58 genes with haplotype differences. These 58 genes were hypothesized as candidate genes for low nitrogen tolerance related to root morphology. Finally, six genes (*Os07g0471300*, *Os11g0230400*, *Os11g0229300*, *Os11g0229400*, *Os11g0618300* and *Os11g0229333*) which expressed differentially in Longjing 31 were defined as more valuable candidate genes for low-nitrogen tolerance. The results revealed the response characteristics of rice to low-nitrogen, and provided insights into regulatory mechanisms of
rice to nitrogen deficiency (Table2).

Table 2: Candidate genes expressed differentially under low nitrogen conditions

Candidate genes	Regulation	QTLs Annotation
Os07g0471300	Down	qRLR7-2AGOprotein
Os11g0230400	Down	qRDR11-1Serpin domain containing protein,putative expressed
Os11g0229300	Down	qRDR11-1 Disease resistance protein RPM1, putative, expressed
Os11g0229300	Down	qRDR11-1 Disease resistance protein RPM1, putative, expressed
Os11g0618300	Down	qLRD11 Protein kinase family protein,putative,expressed
Os11g0229333	Down	qRDR11-1 Hypothetical gene

Among these 493 genes, 174 genes showed different haplotype patterns, with 96 genes having 2 haplotypes, 54 genes having 3 haplotypes, 18 genes having 4 haplotypes, 4 genes having 5 haplotypes, and 2 genes having 6 haplotypes, respectively. According to the phenotypic data of 295 accessions, there were significant phenotype differences among different haplotypes of 58 genes . Therefore, it was hypothesized that these 58 genes were candidate genes for low nitrogen tolerance related to root morphology.

The results showed six favorable alleles including Hap2 of *Os07g0471300*, Hap1 of *Os11g0230400*, Hap3 of *Os11g0229300*, Hap2of *Os11g0229400*, Hap1 of *Os11g0618300* and Hap2 of *Os11g0229333.*

Low-nitrogen tolerance is a complex trait controlled by multiple genes. In recent years, many low-nitrogen tolerance genes have been identified, such as TOND1, OsNAC42, OsNPF6.1 and OsTCP19 (Zhang *et al.,* 2015; Tang *et al.,* 2019; Liu *et al.,* 2021). With the development of high-throughput sequencing technology, the research method combining GWAS and RNA-seq has provided possibilities

to quickly analyze the genetic basis of complex traits, such as lncRNAs response to low-nitrogen, seed germination ability and seedlings root length under drought stress (Guo *et al.,* 2020; Ma *et al.,* 2022). Research also identified many saline-alkaline tolerant candidate genes (OsIRO3, OsSAP16) by integrating GWAS/BSA and transcriptome data analysis (Li *et al.,* 2019; Lei *et al.,* 2020). In this study, the transcriptome of low-nitrogen tolerant variety and low-nitrogen sensitive variety were sequenced. By combining the amount of gene expression, six genes which expressed differentially in low-nitrogen tolerant varieties were defined as more valuable candidate genes among the 58 genes with haplotype differences.

Protien kinases and phosphatases are well known regulatory proteins involved in various signal transduction pathways. Researchers identified at least 6 protein kinase and one protein phosphatase genes that are rapidly induced by −N in rice roots. The only protein phosphatase gene found to be rapidly induced by −N is Os09g0325700 that encodes protein phosphatase 2 C 68 (PP2C68). The rice PP2C68 is a homolog of *Arabidopsis* HAI1/2/3 (highly ABA-induced PP2C protein 1/2/3). It is not clear if ABA can induce the expression of PP2C68. Nevertheless, −N can induce the expression of PP2C68 in rice roots.

Some autophagy-related gene could enhance N remobilization and yield and NUE. For example, the overexpression of OsATG8a in rice could increase shoot biomass, yield, and NUE when transgenic plants were exposed to high N conditions (Yu *et al.,* 2019), while overexpression of OsATG8b led to increased biomass, yield, and NUE at moderate and low N (Zhen *et al.,* 2019). Similarly, the constitutive expression of OsATG8b in rice led to more biomass and higher grain yield under sufficient N conditions (Fan *et al.,* 2020), and the overexpression of OsATG8c led to improvements in shoot biomass, grain yield, and NUE under moderate and low N fertilization (Zhen *et al.,* 2019).

For example, compared with WT, the influx rate of $^{15}NO_3$, agronomic nitrogen use efficiency, and nitrogen recovery efficiency of OsNRT2.1 or OsNAR2.1 overexpression lines increased by 10%, 20%, and 30%, respectively, while the co-overexpression of OsNRT2.1 and OsNAR2.1 lines increased by 40%, 50%, and 60%, respectively. The elite haplotype OsNPF6.1HapB improves NUE under low nitrogen conditions, which was activated by OsNAC42 (Tang *et al.,* 2018). *Indica* OsNR2 promotes NO_3 uptake by feedback interaction OsNRT1.1B, which increased effective tiller number, grain yield, and NUE. Therefore, the co-expression of OsNAC42 OsNPF6.1HapB cascade, *Indica* OsNR2-OsNRT1.1B could improve NUE in rice.

Ammonia Assimilation

Ammonium is combined with glutamate to form glutamine. This reaction is catalyzed by glutamine synthetase (GS or GLN). Plants harbor several isoforms of cytosolic GS (GS1). In rice, there are three genes that encode cytosolic GS1 (*OsGS1;1, OsGS1;2* and *OsGS1;3*), and one gene encodes chloroplastic GS2 (*OsGS2*) (Tabuchi *et al.,* 2007). *OsGS1;1* is mainly expressed in the shoot, *OsGS1;2* mRNA is abundant in the root, *OsGS1;3* mRNA is present only in the spikelets, and *OsGS2* is mainly expressed in the leaf (Tabuchi *et al.,* 2005). Rice GS1;3 is highly expressed in seeds during grain filling and germination, suggesting a unique role in these processes. This study aimed to investigate the role of GS1;3 for rice growth and yield. Tos17 insertion lines for GS1;3 were isolated, and the nitrogen (N), amino acid, and ammonium contents of GS1;3 mutant grains were compared to wild-type grains. The spatiotemporal expression of GS1;3 and the growth and yield of rice plants were evaluated in hydroponic culture and the paddy field. Additionally, the stable isotope of N was used to trace the foliar N flux during grain filling. Results showed that the loss of GS1;3 retarded seed germination. Seeds of GS1;3 mutants accumulated glutamate but did not show a marked change in the level of phytohormones. The expression of GS1;3 was detected at the beginning of germination, with limited promoter activity in seeds.GS1;3 mutants showed a considerably decreased ripening ratio and decreased N efflux in the 12th leaf blade under N deficient conditions. The β-glucuronidase gene expression under control of the GS1;3 promoter was detected in the vascular tissue and aleurone cell layer of developing grains. These data suggest unique physiological roles of GS1;3 in the early stage of seed germination and grain filling under N deficient conditions in rice (Fig.5).

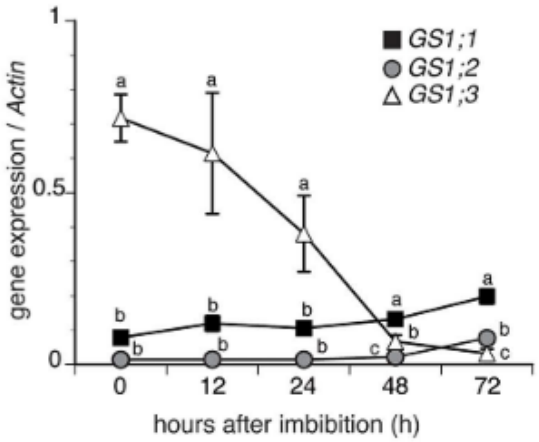

Figure 5: Time course qPCR analysis of *GS1* isoforms in WT plants during rice seed germination after imbibition of mature seeds. Data represent mean ± SD (n = 3) (After Fujita *et al.*, 2022)

Results demonstrate that the co-overexpression of OsGS1;1 and OsGS2 isoforms in transgenic rice plants enhanced its tolerance to osmotic and salinity stress at the seedling stage. The transgenic lines maintained significantly higher fresh weight, chlorophyll content, and relative water content than wild type (wt) and null segregant (ns) controls, under both osmotic and salinity stress. The OsGS1;1 /OsGS2 co-overexpressing transgenic plants accumulated higher levels of proline but showed lower electrolyte leakage and had lower malondialdehyde (MDA) content under the stress treatments. The transgenic lines showed considerably enhanced photosynthetic and agronomic performance under drought and salinity stress imposed during the reproductive stage, as compared to wt and ns control plants. The grain filling rates of the transgenic rice plants under reproductive stage drought stress (64.6 ± 4.7%) and salinity stress (58.2 ± 4.5%) were significantly higher than control plants, thereby leading to higher yields under these abiotic stress conditions. Preliminary analysis also revealed that the transgenic lines had improved tolerance to methyl viologen induced photo-oxidative stress (James *et al.*, 2018).

Systematically analyzed the growth phenotype, carbon-nitrogen metabolic status and gene expression profiles in *GS1;1*-, *GS1;2*-overexpressing rice and wild type plants. It was revealed that the *GS1;1*-, *GS1;2*-overexpressing plants exhibited a poor plant growth phenotype and yield and decreased carbon/nitrogen ratio in the stem caused by the accumulation of nitrogen in the stem. In addition, the leaf SPAD value and photosynthetic parameters, soluble proteins and carbohydrates varied greatly in the *GS1;1*-, *GS1;2*-overexpressing plants. Furthermore, metabolite profile and gene expression analysis demonstrated significant changes in individual sugars, organic acids and free amino acids, and gene expression patterns in *GS1;1*-, *GS1;2*-overexpressing plants, which also indicated the distinct roles that these two *GS1* genes played in rice nitrogen metabolism, particularly when sufficient nitrogen was applied in the environment. Thus, the unbalanced carbon-nitrogen metabolic status and poor ability of nitrogen transportation from stem to leaf in *GS1;1*-, *GS1;2*-overexpressing plants may explain the poor growth and yield.

In rice coleoptile, a tissue highly tolerant to anaerobic stress, it was shownthat GS, ferredoxin (Fd)-dependent GOGAT and Fd-NADP 1 reductase are newly synthezised during the stress for the re-assimilation of ammonia (Mattana *et al.*, , 1994; Mattana *et al.*, , 1996; Mattana *et al.*, , 1997)(Fig. 6). Ferredoxin-dependent glutamate synthase (Fd-Gogat; EC 1.4.7.1) in leaf and root plastids is the last enzyme involved in the pathway of nitrate assimilation in higher plants. *Arabidopsis thaliana* expresses two different genes the first, light regulated, specific of green tissues and the second expressed in other tissues. In this work, it was investigated whether in clone, OsGog2 AC Y12595, this gene is up-regulated by light or it is expressed under darkness. Fd-GOGAT specific activity, protein and mRNA increased after light treatment in rice shoots. In roots, the activity and the protein content remained constant, whereas the mRNA is repressed by light treatment. The results obtained using a specific probe, situated in

the 3' untranslated region of the OsGog2 cDNA, indicated that OsGog2 gene is up-regulated by light and that its expression is tissue specific and suggested that a dark expressed Fd-Gogat gene could be present in rice similarly as in *Arabidopsis*.
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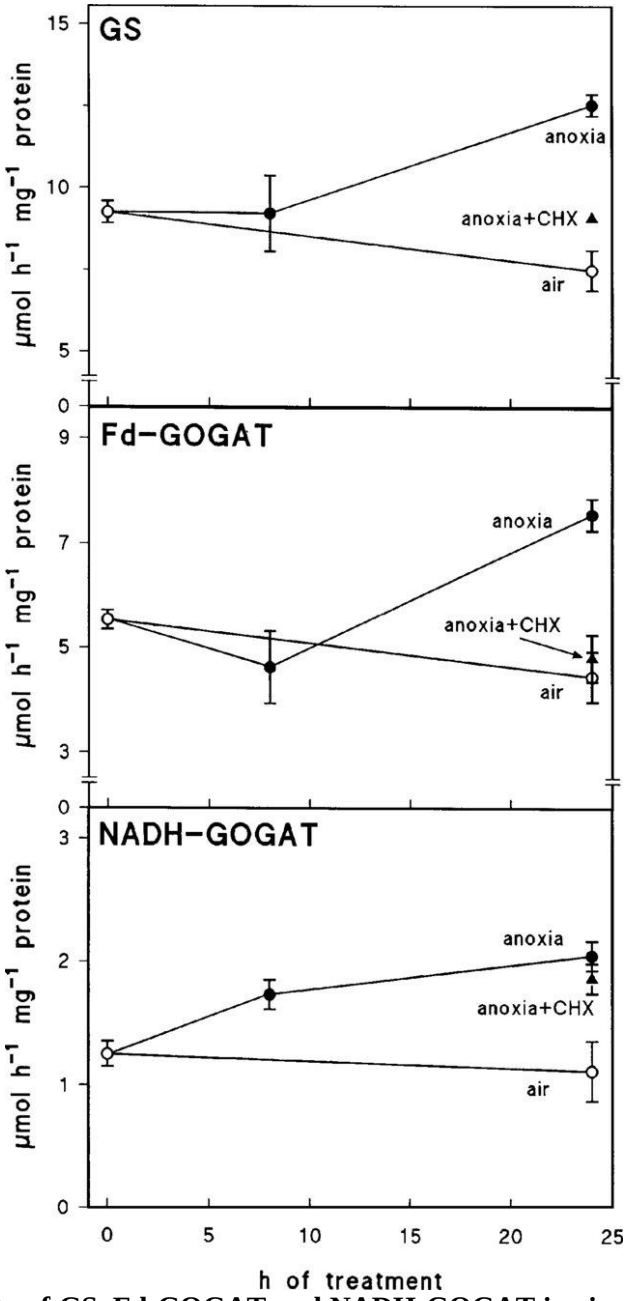


Figure 6: Specific activity of GS, Fd-GOGAT and NADH-GOGAT in rice roots in air or during 24 h of anaerobic treatment. Two µg ml 1 CHX was added to the 24 h-anaerobic treatment. Vertical bars SE; N 5

Amino Acids

It was found that the free amino acids of rice decreased under high and low nitrogen. The analysis of metabolic profile results showed that levels of three aromatic amino acids (Tyr, Trp,Phe), five glutamate family amino acids (Glu, Gln, l-Arg, d-Arg, Pro), two branched-chain amino acids(Lue, Ile), five aspartate family amino acids (Asp, Lys, Thr, Met, D1-homoserine), and three nitrogenous metabolites

(_-ala, citrulline, GABA) were reduced under low N. The levels of three aromatic amino acids (Tyr, Trp, Phe) and two branched chain amino acids (Lue, Ile) decreased under high N, whereas five glutamate family amino acids (Glu, Gln, l-Arg, d-Arg, Pro), three aspartate family amino acids (Asp, Thr, D1-Homoserine), and three nitrogenous metabolites (-ala, citrulline, urea) had increased accumulation under high N. d-Methionine and GABA had decreased accumulation under high N.

Consistent with metabolome profiles,N deprivation decreased the transcript levels of the genes OsGLT1, OsArgA, OsCOA3.1, OsCOA3.2, OsALDH2b1, OsCAD, OsThrA, OsThrC, and OsLysC, and increased the transcript levels of OsTYDC, Agxt2.1, Agxt2.2, OsIlvb, and OsIlve. This result indicated that low N inhibited the amino acid synthesis process and promoted the amino acid degradation process. Excess N increased the transcript levels of OsGLT1, OsArgA, OsLysC, and OsSAT and decreased the transcript levels of OsALDH3H1. Cinnamyl alcohol dehydrogenase, trans-cinnamic acid 4-monooxygenase, and cinnamoyl-CoA reductase are key enzymes in phenylpropanoid biosynthesis. OsCAD8B, OsCAD8C, OsCYP73A, and OsCCR expression were induced by low N, and OsCYP73A expression was inhibited by high N. In addition, the contents of ferulate and sinapate increased under low N and the content of ferulate decreased under high N.

Collectively, these observations indicate that the TCA cycle enhancement enables a more rational allocation of nitrogen for the synthesis of various amino acids and provides more 2-OG enhanced ammonium re-assimilation, to enhance rice adaptation to low-nitrogen stress. The TCA cycle and PPP pathway were inhibited by high nitrogen in the present study, indicating that rice production capacity and carbon skeleton synthesis will be inhibited under long-term high nitrogen stress.

GDH is active in the in vivo catabolism of glutamate and, specifically, it is catabolized to ammonium and 2-oxoglutarate, thus ensuring carbon skeletons for the TCA cycle. McIntosh *et al.,* (1998) confirmed that, under many different environmental conditions, higher plants modify their respiratory metabolism to ensure the turnover of the TCA cycle to continuous supply carbon skeletons for biosynthetic demands, as a regulatory phenomenon, allowing a metabolic response to stresses. As also previously reported (Brandt and Molgaard, 2001; Wang *et al.,* 2008), results suggest that the plants grown in organically managed plots modified their metabolism, not only in terms of glutamate content (significantly lower in the three different organic plots compared to the conventional plot) but also in the higher expression of GDH gene. The main pathway of ammonium assimilation in plants is represented by the GOGAT cycle. Further, results suggest that GDH significantly contributes to secondary ammonium assimilation, playing a complementary role to that of the GS/GOGAT cycle. It has also been recently demonstrated that GDH alleviates ammonium toxicity and suppresses photorespiration by assimilating excess NH_4^+ and disturbing the delicate balance of carbon and nitrogen metabolism, thereby improving drought tolerance in rice (Yan *et al.,* 2021).

Nitrogen Stress

Most of the studies have implicated nitrogen transport genes, nitrogen assimilation genes, and GS/GOGAT cycle genes involved in nitrogen use efficiency of rice (Gutierrez, 2012; Nunes-Nesi *et al.,* 2010; Yang *et al.,* 2016). In a study, it was found that nitrogen transport and assimilation were promoted by low nitrogen, while inhibited by high nitrogen. Interestingly, results showed that the contents of Glu and Gln were inhibited by low nitrogen and promoted by high nitrogen. Previous studies have also shown that Glu accumulation inhibits the expression of nitrate reduction and transport genes (Hoff *et al.,* 1994; Vincentz *et al.,* 2010).

A total of 110 early rice varieties collected from 52 countries (regions) in different geographical regions of the world were comprehensively identified for agronomic traits. It was found that there was a high correlation between tillering nitrogen response ability and NUE variation in many agronomic traits under different nitrogen fertilizer conditions. A variant of OsTCP19 promoter was identified by genome-wide association analysis (GWAS). Further study found OsTCP19 regulated nitrogen uptake by regulating the expression of nitrogen utilization genes to meet the demand of nitrogen growth (Liu *et al.,* 2021). A similar situation occurs in the promoter of OsARE. After analyzing 2155 rice varieties, it was found that 18% of *Indica* and 48% of *Japonica* had small insertion in ARE1 promoter, which led to the decrease of

ARE1 expression, caused delayed senescence, and resulted in 10–20% grain yield increases under limited nitrogen condition (Wang *et al.*, 2018).

Examination of the root DEGs between Pokkali and Bengal showed more genes in Pokkali than in Bengal under N starvation. Likewise, the number of DEGs during early N recovery was also higher in Pokkali than in Bengal roots. It was not just the involvement of more DEGs, but also that the allelic differences resulting from different genetic backgrounds may be responsible for an increased N uptake efficiency in Pokkali compared to Bengal. There is a sizable number of genes overlapping between both genotypes observed in our study compared to an earlier study (Sinha *et al.*, 2018). These common genes may be involved in the inherent mechanisms associated with uptake regardless of genotypic differences.

Nitrogen stress conditions caused NRT2.1 (LOC_Os02g02170) to be profoundly more modulated in Pokkali than in Bengal roots, while the nitrogen assimilation-mobilization genes, such as ammonia transporters, nitrate reductases, glutamine synthetases, and glutamine oxoglutarate aminotransferases (GOGAT), were either unchanged or downregulated under low N conditions (Table 3). The nitrate transporters in Pokkali played a major role in enhancing the uptake of N, particularly under limited N conditions, compared to Bengal. In addition, the N assimilation-mobilization activity was reduced as the latter processes were highly regulated by feedback inhibition depending on the nitrogen availability of plants. The downregulation of nitrate reductase, ammonia transporters, and glutamine synthetase could be due to the regulation of the substrate for nitrogen assimilation, particularly during chronic N stress.

Table 3: Nitrogen uptake and metabolism-related DEGs of Pokkali and Bengal in response to low N and early (1-h) recovery conditions.

Function	MSU ID	Description	Low N Pokkali	Early N recovery	Low N Bengal	Early N recovery
Nitrate Transporters	LOC_Os10g40600 LOC_Os02g02170 LOC_Os02g02190 LOC_Os01g50820	NRT 1.1B NRT 2.1 NRT 2.2 NRT 2.3	- U U U	U U U -	- - U U	U U U -
Amm.tran.	LOC_Os02g40730	AMT 1.2	D	U	-	U
Nitrate reductase	LOC_Os08g36480 LOC_Os08g36500 LOC_Os02g53130	NR 1 NR 2 NADH-NR 2	- - D	- - U	- - D	U U U
	LOC_Os03g12290 LOC_Os01g48960	GS 1;2 NADH-GOGAT	D -	U U	D -	U U

U-Upregulated; D-Downregulated;-no significant difference

The most widely cultivated rice in Asia has two major subspecies, *japonica* and *indica*. In general, *indica* rice cultivars are more sensitive to nitrogen than *japonica* rice cultivars in several aspects, including the plant height, the tiller number, and the biomass (Sun *et al.*, 2014; Hu *et al.*, 2015). Whether ABC1 is potentially involved in the different responses of *indica* and *japonica* rice plants to nitrogen, it was analyzed genetic variations in the ABC1 locus. Analysis of the ABC1 locus by DNA sequencing in 19 rice cultivars, including *japonica* and *indica* was recorded.

An analysis identified 177 single-nucleotide polymorphisms (SNPs) in19 cultivars, of which 31 SNPs were located in the coding sequences of ABC1. Among these 31 SNPs, five caused,non-synonymous or mis-sense substitutions. These five nonsynonymous SNPs showed distinct patterns between *japonica* and *indica* varieties , which were designated as ABC1-ja (Ile-765, Ile-1293, Ala-1302, Gln-1363,Val-1417) and ABC1-in (Val-765, Leu-1293, Val-1302, Pro-1363, Ile-1417).

To gain more insight into the possible differentiation of ABC1 between *japonica* and *indica*, searched additional SNPs in ABC1through the Rice HapMap Project (RHP, http //202.127.18.221/cgi-bin/gbrowse/rhp3/) (Huang *et al.*, , 2012) and RiceVarMap(Zhao *et al.*, , 2015). Strikingly, whereas all 483 *japonica* and the majority (66/67) of aus accessions were the ABC1-ja haplotype, more than 85%

(444/520) of *indica* cultivars showed the ABC1-in haplotype (Fig.). These results indicate that the ABC1locus shows highly and specifically differentiated patterns between *japonica* and *indica*. In addition, a third haplotype in the *indica* group (Val-765, Leu-1293, Ala-1302, Pro-1363, Ile-1417) was identified, which occurred with the highest frequency in wild rice, designated as ABC1-w (Fig.7) (Yang *et al.*, 2016).

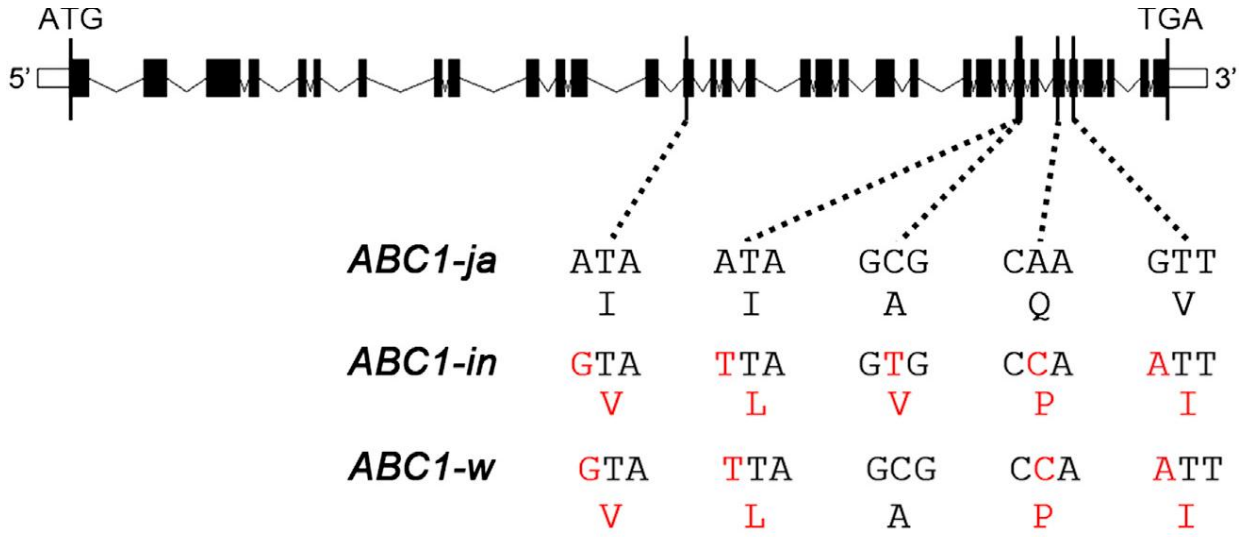
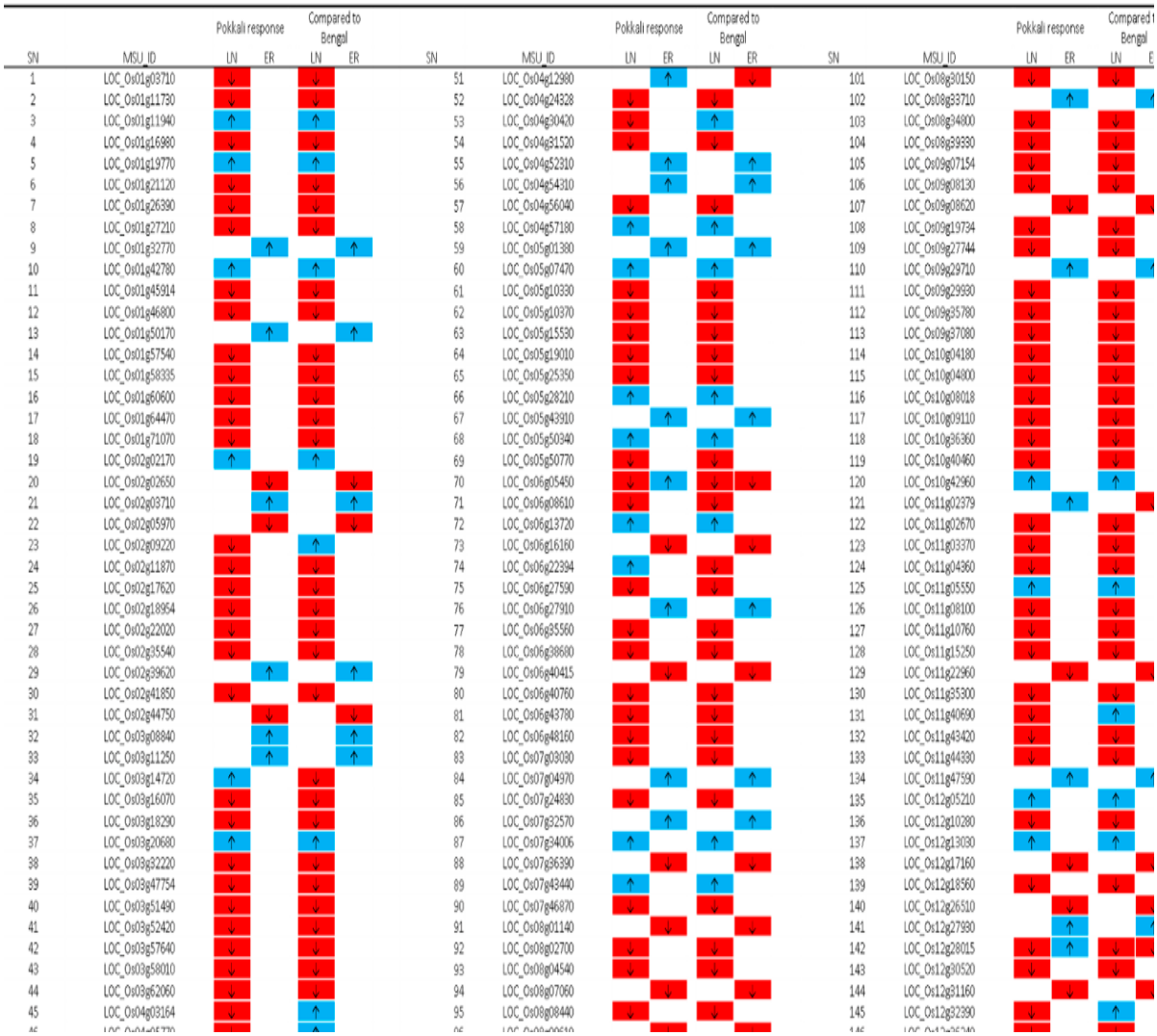


Figure 7: Non-synonymous SNPs in ABC1. ABC1-ja, ABC1-in, and ABC1-w represent haplotypes of ABC1 in all *japonica* cultivars, the majority of the *indica* cultivars, and the haplotype mainly in wild rice, respectively. Variations in ABC1-in and ABC1-w are highlighted in red.

The weak mutant allele abc1-1 mutant shows a typical nitrogen-deficient syndrome, whereas the T-DNA insertional mutant abc1-2 is seedling lethal. Metabolomics analysis revealed the accumulation of an excessive amount of amino acids with high N/C ratio (Gln and Asn) and several intermediates in the tricarboxylic acid cycle in abc1-1, suggesting that ABC1 plays a critical role in nitrogen assimilation and carbon–nitrogen balance. Five non-synonymous single-nucleotide polymorphisms were identified in the ABC1 coding region and characterized as three distinct haplotypes, which have been highly and specifically differentiated between *japonica* and *indica* subspecies. Collectively, these results suggest that ABC1/ OsFd-GOGAT is essential for plant growth and development by modulating nitrogen assimilation and the carbon–nitrogen balance.

The global expression profiles of root tissues collected from low and high N treatments at different time points in two rice genotypes, Pokkali and Bengal, with contrasting responses to N stress and contrasting root architectures were examined. Overall, the number of differentially expressed genes (DEGs) in Pokkali (*indica*) was higher than in Bengal (*japonica*) during low N and early N recovery treatments. Most low N DEGs in both genotypes were downregulated whereas early N recovery DEGs were upregulated. Of these, 148 Pokkali-specific DEGs might contribute to Pokkali's advantage under N stress. These DEGs included transcription factors and transporters and were involved in stress responses, growth and development, regulation,and metabolism(Table 4). Many DEGs are co-localized with quantitative trait loci (QTL) related to root growth and development, chlorate-resistance, and NUE. Findings suggest that the superior growth performance of Pokkali under low N conditions could be due to the genetic differences in a diverse set of genes influencing N uptake through the regulation of root architecture.

Table 4.148 Pokkali-specific root DEGs under low N and early recovery conditions. The expression pattern of these genes is shown for low nitrogen (LN) and 1-h N recovery/early response (ER) in Pokkali as well as compared to Bengal. The upward arrows in blue notes upregulation whereas downward arrows in red notes downregulation.



Carbon-Nitrogen Balance

It was noted that OsDOF11promotes nitrogen uptake and then maintains the ratio of fresh weight to dry weight in seedling plants and the effective leaf blade at flowering stages. Mutants of the sucrose transporter gene OsSWEET14 displayed a phenotype similar to that of OsDOF11.

By microarray analysis and qRT-PCR in OsDOF11 mutant plants, OsDOF11 affected the transcription level of amino acid metabolism-related genes. It was further found that mainly amino acid contents were reduced in flag leaves but increased in seeds. Both sugar and organic nitrogen changes caused the ratio of fresh weight to dry weight to decrease in OsDOF11 mutant seedling plants and mature leaves, which might result in vigorous reduced metabolic activity and become less susceptible to stress. These results demonstrated that OsDOF11 affected nitrogen metabolism by sugar distribution in rice, which provided new insight that OsDOF11 coordinated with C and N balance to maintain plant growth activity.

Calcium

Calcium-dependent protein kinases are involved in various biological processes, including hormone response, growth and development, abiotic stress response, disease resistance, and nitrogen metabolism. Xing *et al.*, (2018) identified a novel mutant of a calcium-dependent protein-kinase-encoding gene, esl4, by performing map cloning. The esl4 mutant was nitrogen deficient, and expression and enzyme activities

of genes related to nitrogen metabolism were down-regulated. ESL4 was mainly expressed in the vascular bundles of roots, stems, leaves, and sheaths.

Salinity

Evaluation of nitrate and ammonium assimilation during reproductive stage in control and salinity ($10dSm^{-1}$ using NaCl) stressed rice plants. Osmotic stress tolerant rice genotype Shabhagidhan (SD) and high yielding yet osmotic and salinity stress sensitive genotype Pusa sugandh-5 (PS5) were evaluated. Salinity stress was given to plants during panicle emergence and flag leaves was collected after 1d, 3d 5d, 7d, 9d,12d and 15d after anthesis. Reproductive stage salinity stress resulted in decrease of membrane stability, relative water content and osmotic potential of rice plants. Reproductive stage salinity stress decreased the expression of nitrate reductase (OsNIA), nitrite reductase (OsNiR), Glutamine synthetase (OsGLN1.1, OsGLN1.2, OsGLN2) and glutamate synthase/GOGAT (OsFd-GOGAT, OsNADH-GOGAT) in flag leaves. In response to stress, SD showed better stress tolerance than PS5 in terms of higher yield stability. Variety SD showed higher leaf nitrate and ammonium content and maintained comparatively higher nitrate and ammonia metabolism enzyme activity than PS5. Salinity stress upregulated the activity of glutamate dehydrogenase enzyme and indirectly contributed to the higher proline content and maintenance of favourable osmotic potential in SD. Expression of *GS2* which has role in photo respiratory ammonia assimilation was upregulated by salinity stress in PS5 in comparison to SD. Rice genotype showing better induction of nitrogen assimilatory genes will be more tolerant to reproductive stage salinity stress.

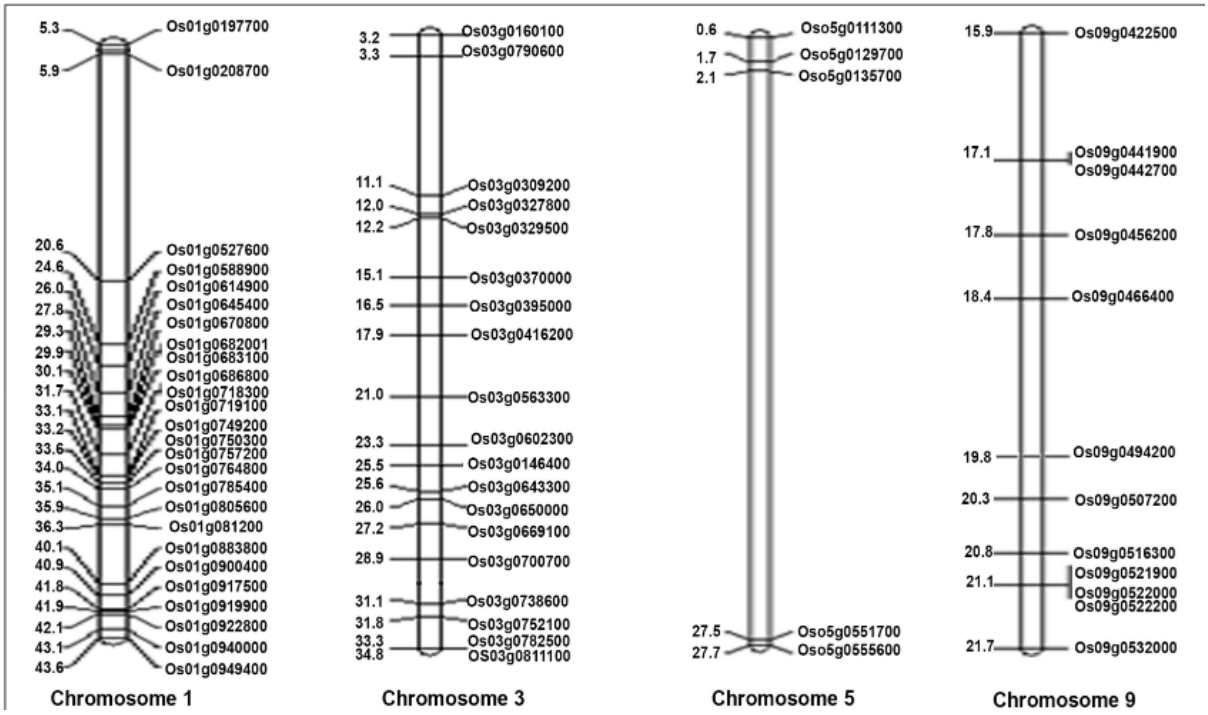


Figure 7: Physical positions of the 62 NUE-candidate genes on four rice chromosomes (chromosomes 1, 3, 5, and 9). Gene ID is given on the right side of the map, and the physical location of genes is given on the left side of the map (in mb).
(After Kumari *et al.*, 2021)

Drought

Under the same drought severity, tolerant variety (DA8) accumulated higher proline than sensitive variety (Malagkit Pirurutong). The expression of P5CS1 and P5CS2, responsible for the synthesis of P5CS enzyme, which catalyzes the first step of the proline synthesis, were induced by drought stress. DA8 showed higher expressions of P5CS2, P5CR (which codes enzyme converting P5C into proline) and OsOAT (involve in proline synthesis through ornithine) than those in Malagkit Pirurutong under severe drought condition. The results suggested that high expression of P5CS1, P5CS2, P5CR and OsOAT genes, particularly under severe drought condition, were in line with the high accumulation of proline. At 4 days after re–watering, proline was quickly degraded due to the decreased expression of synthesis genes and the increased expression of P5CDH, a gene related to proline degradation after re–watering.

Nitrogen-use efficiency is not a biological measure by itself, but a derivative of biological measures such as the N-response and yield. Many genes involved in the yield or N-response are known separately, but a comprehensive listing and analysis of genes that are both N-responsive and yield-related were not available. Therefore, it was identified 1,064 common genes between 14,791 N-responsive genes and 1,842 yield-related genes known in rice and analyzed them. These 1,064 genes were termed as NUE-related genes and were used for further downstream analyses. As these genes were derived from transcriptomic analyses using different N-forms, separate Venn selections revealed the breakup of yield-related genes for each N-form. The highest numbers of yield-related genes were found for nitrate, followed by ammonium, ammonium nitrate and urea. Nevertheless, all 1,064 genes are of interest for further molecular characterization of NUE, especially considering that field soils tend to contain dynamic mixtures of multiple N-forms. Their chromosomal localization revealed that most of these were present on chromosome 3, followed by chromosomes 1, 2, and 4, while chromosome 12 harbors the least number of genes. Further, chromosomes 1–8, 10, and 11 contain more N-upregulated genes in comparison to N-downregulated genes, while chromosomes 9 and 12 have more N-downregulated genes in comparison to N-upregulated genes (Fig. 7).

CONCLUSION

Using gene editing by CRISPR-Cas9 technology to change the expression pattern of some genes is a choice for precision agriculture. Nitrate transporters can be effectively used to improve crop yield and through genome editing. Changing the expression of some nitrate transporters or their regulators can improve the NUE in crops. However, it is not the best way to improve NUE by simply aggregating single positive alleles in rice. The overexpression of multiple genes at the same time can improve the nitrogen use efficiency of more rice.

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