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Improving nitrogen use efficiency in crop production

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Molecular interventions for improving crop nitrogen use efficiency: trends, opportunities and challenges in rice

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1 Introduction

Reactive nitrogen (N) is an indispensable macronutrient nutrient, which is part of important biomolecules that constitute both structural and non-structural parts of the cells. Soil contains a mixture of N forms, in which the most abundant inorganic N forms are nitrate and ammonium, whereas amino acids and peptides are the organic N forms. In Asia, the most widely used N fertilizer is urea, which can be utilized either directly by plants or in the split N forms (ammonium or nitrate) produced by bacterial nitrification in the soil. Reactive N regulates many physiological, biochemical, and molecular processes; therefore crop yield depends on N fertilizer input and the biological N use efficiency (NUE) of plants. These two parameters significantly contribute to the effective utilization of reactive N (Ladha et al., 1998; Raghuram and Sharma, 2019; Lee, 2021; Madan et al., 2022). Low crop NUE results in inefficient utilization of N fertilizers, and the unused N fertilizers cause air and water environmental pollution and climate change, apart from economic wastage

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(Sutton et al., 2013, 2021; Raghuram et al., 2021). The recognition that reactive N pollution adversely affects biodiversity, human health and climate change (Sutton et al., 2013, 2019) led governments to adopt resolutions on sustainable N management (UNEA4.14, 2019; Raghuram et al., 2021; Sutton et al., 2021). Recently, articles by Ladha et al. (2020) and Winiwarter et al. (2022) have comprehensively discussed on reactive N and UN sustainable development goals (SDGs). We need to improve crop NUE by manipulating the biological system and/or reduce the consumption of N fertilizers as a part of our wider plans for sustainable N management, in order to achieve all the relevant SDGs (Ladha et al., 2020).

Rice is considered a model crop plant and has many advantages over wheat and maize as the rice genome is smaller and was sequenced much earlier, offering many genetic and genomic resources as compared to other crops. Further, the improved rice transformation efficiency as compared to wheat and maize expedites the molecular studies in rice. Rice has diverse genetic resources, including locally adapted cultivars, wild relatives and landraces among the cereals. According to the Global Crop Diversity Trust (<https://www.croptrust.org/>), established in 2004 by the Food and Agriculture Organization, over 500 000 accessions of cultivated rice and wild relatives are known in the world, and the International Rice Research Institute holds over 130 000 rice accessions (Jamora and Ramaiah, 2022). Further, rice is the only crop in which genomic diversity is well characterized on over 3000 genomes (Li et al., 2014).

An increase in the application rate of N fertilizers improves crop yield up to a certain extent, but after that, the addition of extra N fertilizers does not increase the crop yield. Over half of the global N fertilizers are consumed by just three crops – rice, wheat and maize with 16% for rice alone (Ladha et al., 2016), though in India it is as high as 37% (Abrol et al., 2017). Yield-centric crop improvement programmes under high input conditions and the lack of low input programmes meant that NUE declined disproportionately even as yields increased slightly (Sutton et al., 2013; Abrol et al., 2017; Raghuram et al., 2022a; Kabange et al., 2022). Many rice varieties such as green revolution variety and super hybrid rice have been developed, but they are responsive to high N fertilizer input (Wang et al., 2021a; Wang and Peng, 2017). Therefore, it is very important to reduce N fertilizer usage while maintaining a similar or high yield, which can be achieved by improving the NUE of rice. Nitrogen processes such as N acquisition from the soil, translocation, assimilation and remobilization are affected by environmental conditions, which make NUE a highly complex trait (Ladha et al., 1998). To dissect the molecular aspects of NUE, many N-responsive or NUE genes associated with above-mentioned processes have been identified in rice (Sharma et al., 2022, 2023; Madan et al., 2022; Kumari et al., 2021; Sharma et al., 2020; Mandal et al., 2022; Pathak et al.,

2020; Sinha et al., 2020; Kumari and Raghuram, 2020; Raghuram and Sharma, 2019; Mandal et al., 2018).

Identification of NUE donor genotypes and associated information are necessary to expedite the rice breeding programmes. For this purpose, we must develop the molecular markers associated with NUE candidate genes for introgression into different rice varieties. In this context, we must also identify the allelic diversity responsible for NUE within rice subspecies germplasms. Quantitative trait loci (QTL) mapping and genome-wide association study (GWAS) have helped to identify the gene(s) and genomic regions responsible for NUE. In this chapter, we have covered these aspects for a better understanding of NUE in rice, with the ultimate goal of achieving rice genotypes with high yield at low N input to save both economic and environmental costs.

2 Definition and selection of nitrogen use efficiency indices

Ratio of input and output has been defined in different ways to calculate the agronomical performance of a crop under different N conditions (Ladha et al., 2005). NUE is the most important biological indicator to reduce N fertilizer input with similar or higher yield. NUE is a complex trait as various phenotypic, physiological, biochemical and molecular changes occur in plants to regulate reactive N homeostasis in plants. It is well known that even cultivars of the same species have significant difference in NUE, which reflects the complex nature of NUE (Ladha et al., 1998). Due to such complexity, various definitions have been proposed to define the NUE and the findings were summarized in different publications (Ladha et al., 2005; Pathak et al., 2008; Raghuram and Sharma, 2019; Briat et al., 2020; Lemaire and Ciampitti, 2020; Congreves et al., 2021; Ciampitti et al., 2022; Ciampitti and Lemaire, 2022; Madan et al., 2022). Congreves et al. (2021) have provided a comprehensive list of known NUE indices including their strength and weakness based on fertilizer, plants, soil and isotopes. To rank the N-efficient rice genotypes, a field study was conducted using medium- and long-duration rice and various agronomically important parameters such as physiological NUE, agronomic NUE and N productivity index (NPI) among others were measured. Analyses revealed that NPI was one of the most important parameters that can be used to rank the N-efficient rice genotypes (Singh et al., 1998). Recently, redefinition of NUE has been proposed (Ciampitti et al., 2022) in the context of N uptake efficiency and N conversion efficiency. Application of N fertilizer (N_f) maximizes the grain yield (Y_{max}), which is related to environmental N losses. To achieve Y_{max} , we must regulate N_f and contribution by soil N (N_s) in such a way that minimum N losses occur to the environment. They also proposed that previously known Nitrogen Nutrition Index (NNI), which is used to measure the plant and crop N status

Table 1 List of common NUE indices used in rice

Important NUE related indices	Abbreviation (unit)	Formula	Outcome
Nitrogen use efficiency	NUE (kg yield kg ⁻¹ N)	$= \frac{\Delta Y (or W)}{\Delta(Nf + Ns)}$	Maximizing grain yield driven by N fertilizer and soil N, while minimizing environmental N losses
Nitrogen nutrition index	NNI	$= \frac{PNC}{Nc}$	This reflects plant N status as the Nc changes throughout plant's growth. NNI < 1 (deficient plant N status), NNI = 1 (optimal plant N status), NNI > 1 (excess N status)
Nitrogen use efficiency (crop)	NUE _{crop} (kg yield kg ⁻¹ N)	$= \frac{Yield Nf}{Fertilizer N}$	Yield per unit of applied N fertilizer.
Partial-factor productivity	PFP (grain yield/ total N)	$= \frac{Yield f}{Fertilizer N}$	The fraction of N fertilizer utilized and allocated to yield N.
Agronomic efficiency	AE (kg grain kg ⁻¹ N)	$= \frac{Yield f - Yield 0}{Fertilizer N}$	The contribution of N fertilizer in yield.
Fertilizer-N recovery efficiency	REfertN (kg N in grain kg ⁻¹ N applied)	$= \frac{Plant Nf - Plant N0}{Fertilizer N} \times 100$	The percent of N fertilizer used by plants.
Physiological efficiency	PE (kg grain yield increase kg ⁻¹ N taken up)	$= \frac{Yield f - Yield 0}{Plant Nf - Plant N0}$	N fertilizer contribution from the plant tissues towards the yield.
N uptake efficiency	NUpE (kg N kg ⁻¹ N)	$= \frac{Plant N}{Fertilizer N + soil N} \times 100$	The percent of soil N utilized by the plant.
N utilization efficiency	NUtE (kg grain kg ⁻¹ N)	$= \frac{Yield}{Plant N}$	N fertilizer contribution from the plant tissues towards the yield irrespective of background N.

(Continued)

Table 1 (Continued)

Important NUE related indices	Abbreviation (unit)	Formula	Outcome
Nitrogen use efficiency (yield)	NUE_{yield} (kg grain kg^{-1} N)	$NUpE \times NUtE$	Basic NUE definition, which predicts the contribution of applied N to the yield N.
N harvest index	NHI (%)	$= \frac{\text{Yield N}}{\text{Plant N}} \times 100$	The fraction of plant tissue N present in yield (Percentage form).
Recovery efficiency of N fertilizer	^{15}NRE (%)	$= \frac{\text{TNdff in Plant or Soil}}{\text{Fertilizer N Applied}} \times 100$	The percent recovery or utilization of N fertilizer.

Adapted from: Ladha et al., 2005; Pathak et al., 2011; Raghuram and Sharma, 2019; Congreves et al., 2021; Madan et al., 2022; Ciampitti et al., 2022.

f, applied fertilizer; 0, not applied fertilizer condition; Plant N_f, amount of N in a fertilized plant; Plant N₀, indicate the weight of a fertilized plant; TNdff, total N derived from fertilizer. N_f, application of N fertilizers; N_s, N contribution by soil; ΔY , yield difference ($Y_{\text{max}} - Y_0$); Y_{max} , maximizing grain yield; Y_0 , grain yield contributed by N_s; W_{max} , maximizing biomass; PNC, plant nitrogen concentration; N_c, minimum PNC that can produce maximum aboveground biomass.

(Lemaire et al., 2008). The NNI index is the ratio between plant N concentration and critical N concentration, which reflects the minimum N uptake required for maximum aboveground biomass. The NNI has been used for estimation of N requirement, N partition, photosynthesis capacity and NUE (Hu et al., 2014; Zhao, 2014; Briat et al., 2020). Commonly NUE indices used for rice experiments are provided in Table 1, and specific NUE indices may be chosen based on the purpose of the experiments. An important consideration in this regard is the most abundant N form(s) available to the plant and the role of soil/root microbiomes associated with different rice subspecies and genotypes (Zhang et al., 2019; Ladha and Reddy, 2019).

3 Nitrogen management practices for improved nitrogen use efficiency

Agronomical practices play an important role in NUE improvement as they reduce N loss occur through volatilization, leaching and denitrification (Udvardi et al., 2021; Ladha et al., 2020). With advancements in technology, N fertilizers have been modified and customized for enhanced NUE. Experiments performed with graded levels of customized fertilizer have been shown to improve NUE in rice (Nagabovanalli Basavarajappa et al., 2021). To achieve maximum yield, farmers apply excess N fertilizers in fewer doses whereas plants need nutrition in

small amounts in many doses. As a result, most of the applied fertilizers are not utilized by the plants bringing down the NUE. To overcome this problem from the fertilizer angle, slow-release fertilizers have been developed incorporating urease and/or nitrification inhibitors with some success for some crops in some countries where farmers could afford their high costs (Li et al., 2018). Recently, nanotechnology has been applied to further improve slow-release fertilizer formulations and its potential for NUE remains to be firmly established (Sahu et al., 2022). Biochar, a partial pyrolysis product of agricultural organic material, has been shown to improve crop efficiency by enhancing moisture retention, nutrient holding capacity of soil, nutrient availability and uptake. It has been shown that the application of biochar improved NUE up to 12% in rice (Liu et al., 2022d). Further, biochar-based N fertilizers such as biochar-coated urea improved NUE by reducing N loss from soil and increasing N uptake in oilseed rape (Jia et al., 2021). The NUE-enhancing properties of biochar-coated urea are yet to be established in rice. Bio-fertilizers, the beneficial microorganisms, are involved in the mineralization process to increase the availability of nutrients and enhance nutrient acquisition in plants. Diazotrophs convert reduced N_2 to ammonia, which is utilized by plants. Biological nitrogen fixation (BNF) helps in NUE improvement in cereals including rice (Ladha et al., 2022). Precision farming such as geographic information systems, remote sensing and variable rate applicators are the technological advancements for NUE improvements. Further, leaf colour chart, chlorophyll meter and drip fertigation help to provide N fertilizer in the right amount at the right time, which reduce N loss and also improve NUE in rice. A more detailed discussion of N management practices is beyond the scope of this chapter, due to its focus on molecular aspects in rice.

4 Biological intervention for nitrogen use efficiency improvement

The various N management practices operate within the inherent genetic capacity of the plants to use available N inputs, which needs to be improved in terms of uptake, utilization and remobilization efficiencies, minimally influenced by agro-climatic conditions (Mandal et al., 2018; Raghuram and Sharma, 2019; Sinha et al., 2020; Kumari and Raghuram, 2020; Moring et al., 2021; Madan et al., 2022). The continuous variability of NUE in the germplasm of every crop is a piece of firm evidence that it is a quantitative trait involving many genes regulating the underlying physiological, biochemical and molecular interactions. Genetic improvement efforts need germplasm diversity for phenotypic characterization/selection and/or identification of donor genotypes for breeding, quantitative trait loci, marker development or genome-wide association mapping. The availability of huge germplasm diversity in rice and advancements in its genomics, functional genomics and biotechnological

tools provide immense opportunities to employ reverse genetics for NUE enhancement in rice (Mandal et al., 2018; Raghuram and Sharma, 2019).

4.1 Nitrogen use efficiency phenotypes

Phenotype is a set of observable and ideally measurable characteristics associated with an inherited trait such as plant NUE, whether morphological, physiological or biochemical, but stable in nature and genetically regulated. Plants of the same genotype interacting with the environment may manifest as different phenotypes, but different genotypes that vary in the extent of the same phenotypic traits are of particular interest in crop improvement for NUE. The NUE is a complex trait, and therefore the identification and characterization of the phenotype and the associated genomic regions/loci/genes is an important but unfinished task in most crops including cereals (Hawkesford and Griffiths, 2019), despite the recent progress in rice (Sharma et al., 2018, 2021; Kumari et al., 2021). The N influences plant growth traits throughout the developmental processes in rice, from germination to seed setting (Sharma et al., 2018, 2021). The root is the primary organ for N uptake, and it has been shown that either N deprivation or excess N causes significant changes in the root architecture of rice (Zhang et al., 2020a; Sharma et al., 2018). Fluctuation in N input causes poorly developed plants with altered tiller number and reduced grain yield (Wang et al., 2017). Further, it has been shown that yield components such as panicle development and grain filling are also influenced by N. Using yield as major differentiator between N response and NUE in rice, we found that out of 20 significantly N-responsive traits, only 6 were significantly correlated with NUE for nitrate or urea and 2 others were N-form specific (Sharma et al., 2021). They include germination, root length, flowering time, shoot length before and after harvest, total plant height, fresh biomass and dry biomass. Details of phenotypic traits are provided in Table 2. Such detailed characterization of the NUE phenotype will boost the identification of highly efficient NUE QTLs and/or the most important candidate genes, which can be used to expedite the NUE improvement programmes in rice.

4.2 Quantitative trait loci mapping, whole genome re-sequencing and genome-wide association study for nitrogen use efficiency candidates

Natural variations exist among the germplasms and their screening leads to the identification of robust and reproducible phenotypes as well as the isolation of alleles responsible for the natural phenotypes (Ogura and Busch, 2015). Identification of stable NUE QTLs, which are less influenced by genotype \times environment interaction is the most important step for NUE improvement in

Table 2 Segregation and shortlisting of phenotypic parameters for N response, yield and NUE

No.	N-responsive parameters	Yield-related parameters - low N	N use efficiency parameters
1	^(a,b,c) Days to germination (G)	^(a,b,c) Days to germination (G)	^(a,b,c) Days to germination (G)
2	^(a, c) Chlorophyll content (V1)	^(a,b,c) Shoot length before harvest (V7)	^(a,b,c) Shoot length before harvest (V7)
3	^(a,b,c) Green leaf number at veg. stage(V2)	^(a) Total leaf number at flowering (V8)	^(a,b,c) Fresh biomass (V11)
4	^(b,c) Yellow leaf number at veg. stage (V3)	^(a,b,c) Fresh biomass (V11)	^(a,b,c) Shoot length aft. harvest (V12)
5	^(a,b,c) Total leaf number at veg. stage (V4)	^(a,b,c) Shoot length after harvest (V12)	^(a,c) Root length (V13)
6	^(a,b,c) Leaf width(V5)	^(a,b,c) Root length(V13)	^(a,b,c) Total plant height (V14)
7	^(a,b,c) Stem thickness (V6)	^(a,b,c) Total plant height (V14)	^(a,b,c) Dry biomass (V15)
8	^(a,b,c) Shoot length before harvest (V7)	^(a,b,c) Dry biomass (V15)	^(a,c) Days to flowering (R1)
9	^(a,b,c) Total leaf number at flowering (V8)	^(a,c) Days to flowering (R1)	
10	^(a,b,c) Green leaf number at flowering (V9)		
11	^(a,b,c) Yellow leaf number at flowering (V10)		
12	^(a,b,c) Fresh biomass (V11)		
13	^(a,b,c) Shoot length after harvest (V12)		
14	^(a,b) Root length (V13)		
15	^(a,b) Total plant height (V14)		
16	^(a,b,c) Dry biomass (V15)		
17	^(a,c) Days to flowering (R1)		
18	^(a,c) Unfilled seed weight (R2)		
19	^(a,c) Total seed weight (R3)		
20	^(ns) Filled seed weight (R4)		
21	^(ns) Panicle weight (R5)		
22	^(ns) Filled seed number (R6)		
23	^(ns) Total seed number (R7)		
24	^(a) Unfilled seed number (R8)		
25	^(a,b,c) Weight of panicle remains (R9)		

This table was adapted from our earlier publication (Sharma et al., 2021). Twenty of the 25 parameters were confirmed as N responsive based on the significance test and shown in the first column. Ten of these parameters shortlisted from feature selection and network analyses were subjected to correlation analysis with grain yield in normal and low N and those parameters that significantly correlated with yield at low N are shown in the second column. The NUE was calculated as yield per unit N input for all genotypes/treatments, and their correlation with each of these ten parameters was analysed. Those that significantly correlated with NUE are shown in the third column. The superscripted alphabet a/b/c over the parameter in the first column denotes parameter in combined nitrate and urea N, only nitrate N and only urea N.

G, germination parameter; ns, non-significant parameter; R, reproductive parameter; V, vegetative parameter.

rice. Many studies have been performed to identify the QTLs/genomic regions and or genes involved in NUE in rice (Table 3). Almost two decades ago, Yamaya et al. (2002) focused on N recycling (sink and source relation) and observed the overexpression of glutamate synthase (NADH-GOGAT from Japonica) in Kasalath, an indica cultivar, increased the grain weight (up to 80%). Since Indica cultivars contain lower NADH-GOGAT protein content in the sink organ as compared to Japonica, overexpression of Japonica NADH-GOGAT confirmed their role in sink regulation. Then, they used the protein content of GS1 (senescing leaf blade) and NADH-GOGAT (developing leaf blade), performed QTLs mapping and identified QTLs associated with N recycling and various agronomic traits. With the advancement of genome information and mapping tools, many NUE QTLs and associated candidate genes have been identified in rice (Table 3). Phenotypic variation is the primary requisite for QTLs identification. Most common phenotypic traits in QTL analyses were chlorophyll content, plant height, tiller number and traits associated with the reproductive phase such as panicle number, seeds and yield. In the context of these traits and various physiological parameters, the N content of plant/organ/tissue and N fertilizer input were used to define the various efficiencies including NUE. Phenotypic parameters used in earlier studies are not defined as phenotypes for NUE. Recently, comprehensive phenotypic analyses established the phenotypes for NUE in rice (Sharma et al., 2021). QTL identification using these defined NUE phenotypes may produce stable QTLs, which are not influenced by genotype \times environment interaction. In many studies of QTL mapping, the identification of candidate genes/loci is hampered by a lack of completely sequenced genome information and a long timeline to accomplish the different steps involved in the experiments. In this context, GWAS have produced many NUE candidates within a short duration and less effort. The genes OsTCP19, OsNAC68 and OsMYB61 among others are the important candidates identified by GWAS in rice (Tables 3 and 4). Though GWAS produce candidate gene(s), their role in NUE must be validated in field condition using transgenic or genome-editing (CRISPR/Cas9) approaches.

4.3 Genetic manipulation of genes involved in nitrogen use efficiency

Functional characterization of a particular biological response may be achieved using forward and reverse genetics approaches. Studies using forward or reverse genetics have provided a wealth of information on NUE in crop plants (Zhang et al., 2020b; Neeraja et al., 2021; Liu et al., 2022a). Forward genetics begins with identified phenotypes and then the isolation of loci/

Table 3 List of QTLs and genomic regions for NUE in rice

Method	Traits	QTL information	Potential candidate gene/marker information	Reference
QTL mapping	EPN, GNPP	<i>qEPN-LN4/qEPN-HN3</i> , <i>qGNPP-LN3</i> <i>qGNPP-LN6</i>	<i>qEPN-LN4/qEPN-HN3</i> (SLR1, Os03g0707600), (Os03ZIP59, Os07g0209800)	Liu et al. (2022b)
GWAS	Chlorophyll content (SPAD value)	<i>n-p8</i> , <i>qSdw8</i> , <i>n-r3</i> , <i>qNCP-3-1</i> <i>Q6</i> , <i>Q31</i> , <i>qRW6</i> <i>qYd6</i> , <i>qSdw6</i> , <i>Q31</i> , <i>qRW6</i>	q1 (LOC_Os08g07010, LOC_Os08g07620, LOC_ Os08g07630, LOC_Os08g07660), q2 (LOC_Os03g10980, LOC_Os03g11030, LOC_ Os03g11190, LOC_Os03g11250), q3 (LOC_Os05g44900, LOC_Os05g45020, LOC_Os05g45030, LOC_Os05g45140, LOC_ Os05g45380, LOC_Os05g45410), q4 (LOC_Os06g43030, LOC_Os06g43090, LOC_Os06g43150, LOC_Os06g43304, LOC_Os06g43384), q5 (LOC_Os06g44250, LOC_Os06g44360, LOC_Os06g44690, LOC_Os06g44750, LOC_Os06g45020)	Li et al. (2022b)
GWAS	-	-	Cytosolic glutamine synthetase 1;1 (OsGS1;1, Os02g0735200)	Liu et al. (2022c)
WGR	Large-grain, yield and NUE	<i>Gs3 allele</i> (nonsense mutated <i>GS3</i> gene)	Grain size 3 (<i>GS3</i> , Os03g0407400)	Yoon et al. (2021)
GWAS	NUE	<i>tld1-D</i>	Auxin-responsive GH3 gene (Os11g0528700)	Rakotoson et al. (2021)
GWAS	TRN and NUE	-	OsTCP19	Liu et al. (2021b)
GWAS	N absorption and utilization	-	OsNAC68	Xin et al. (2021)

GWAS	Plant height, tiller number, chlorophyll content and leaf length	-	MYB61 (Os01g0285300), MOC2 (Os01g0866400), OsOAT (Os03g0643300), OsCIPK04 (Os12g0603700)	Lu et al. (2021)
GWAS	NUE	-	OsNLP4 (Os09g0549450)	Yu et al. (2021)
QTL mapping	Grain nitrogen increase rate, agNUE	qGIR-11, q-N8_agNUE-1, q-N8_agNUE-6, q-N18_agNUE-11	qGIR-11 (chr 11, Start bp 4,068,737 and end bp 4,236,048), q-N8_agNUE-1 (Chr 1, Start bp 3,805,999 and end bp 4,740,546), q-N8_agNUE-6 (chr 6, Start bp 25,153,480 and end bp 25,272,880), q-N18_agNUE-11 (chr 11, Start bp 3,936,668 and end bp 4,083,717)	Bai et al. (2021)
QTL mapping	NUE	qSBM1	SBM1 (LOC_Os01g65120)	Xu et al. (2021)
QTL mapping	NDT and NUE	-	The bins 16, 1301, 1465, 1486, 3464 and 6249 shared the QTLs or genes for NDT identified in this study and NUE	Shen et al. (2021)
QTL mapping	NUE	qNLA1 and qNLA2	MYB61 (LOC_Os01g18240)	Gao et al. (2020)
QTL mapping	NUE	qDNR1	DNR1 (LOC_Os01g08270)	Zhang et al. (2021)
QTL mapping	PNUE ANUE	-	Markers RM13181-RM13201 covering 467.706 kb has 42 candidates	Vishnukiran et al. (2020)
QTL mapping	NUE	qCR2	OsNR2 (Os08g0468700)	Gao et al. (2019)
GWAS	NUE	-	OsNPF6.1 (LOC_Os01g01360)	Tang et al. (2019)

(Continued)

Table 3 (Continued)

Method	Traits	QTL information	Potential candidate gene/marker information	Reference
Association mapping	Yield under low N	qGYN5-1 (Os05g0208000) qGYN7 (Os07g0617800) qGYN10 (Os10g0189600)	2-oxoglutarate/malate translocator (Os05g0208000), alanine aminotransferase (Os07g0617800) and pyridoxal phosphate-dependent transferase (Os10g0189600)	Rao et al. (2018)
QTL mapping	NUE	qngr2	NGR2/GRF4	Li et al. (2018)
QTL mapping	NUE	qNUE6	Peptide transporter PTR2 (Os06g0264500) and Asparagine synthetase (Os06g0265000)	Yang et al. (2017)
QTL mapping	NUE	qNUE2.1 qNUE4.1 qNUE6.1 qNUE6.2 qNUE70.1 qNUE70.2	-	Zhou et al. (2017)
QTL mapping	aNUE pNUE agNUE	-	aNUE (RM055-RM3199 on chr3, RM433-RM230 on chr8) pNUE (RM287-RM209 on chr11) agNUE (RM055-RM3199 on chr3, RM433-RM230 on chr8)	Nguyen et al. (2016)
QTL mapping	Agricultural NUE	qANAE4	-	Dai et al. (2015)
QTL mapping	NUE	qCHR10	NRT1.1B/OsNPF6.5 (LOC_Os10g40600)	Hu et al. (2015)
QTL mapping	NUE	TOND1	TOND1 (LOC_Os12g43440)	Zhang et al. (2015) ^a
QTL mapping	Grain yield per plant, spikelets per panicle, number of filled grains per panicle, number of panicles per plant	qGYP-4, qSP-1a, qSP-3b, qFGP-1a, qFGP-3a, qFGP-8a, qPNP-3a	qGYP-4 (RM273-RM241, ch4), qSP-1a (RM1-RM3746, chr1), qSP-3b (RM135-RM168, chr3), qFGP-1a (RM1-RM3746, chr1), qFGP-3a (RM135-RM168, chr3) qFGP-8a (RM5556-RM310, chr8), qPNP-3a (RM135-RM168, chr1)	Yue et al. (2015) ^a

QTL mapping	NUE	qNGR9	Dense and erect panicle 1 (DEP1, Os09g0441900)	Sun et al. (2014)
QTL mapping	NDT and NUE	qPE3, qPE7 and qPE11	-	Wei et al. (2012)
QTL mapping	NUE	qNUE1, qNUE11	Four genomic regions, including C86-C2340 on chromosome 1, RZ599-R1738 on chromosome 2, RZ471-C1023 on chromosome 7 and R3203-RM20a on chromosome 11, were found to contain QTLs for both NUE and GY.	Wei et al. (2011)
QTL mapping	Relative panicle number and relative yield	Orpn-2a, Orpn-2b, Oryd-7a, Oryd-10	Relative panicle number [Orpn-2a (RM211, chr 2), Orpn-2b (RM322-RM424)], relative yield [Oryd-7a (RM542, chr7), Oryd-10 (RM467, chr10)]	Wang et al. (2009)
QTL mapping	NUE	qNUE-3	A QTL region between markers RZ678, RZ574 and RZ284 was associated with nitrogen use and yield.	Senthivel et al. (2008)
QTL mapping	pNUE	pnue9	Marker interval S9093-S9073	Cho et al. (2007)
QTL mapping	Plant height, tillers number per plant, chlorophyll content (low and N0 conditions).	-	QTL for plant height (Rh4118-RM248 on chr7), QTLs for tillers number per plant (RM401-RM417 on chr4 and OSR31-RM55 on chr3), QTL for chlorophyll (RM324-RM26 on chr7)	Tong et al. (2006)
QTL mapping	Protein contents of GS1 (senescing leaf blade) and NADH-GOGAT (developing leaf blade)	-	Glutamine synthetase (GS1, Os02g0735200) and glutamate synthase (NADH-GOGAT, Os01g0681900)	Yamaya et al. (2002)

The QTLs were identified using multiple phenotypic traits, and we considered those associated with yield and/or NUE. agNUE, agronomic nitrogen use efficiency; ANUE, agronomic nitrogen use efficiency; ARE, agricultural nitrogen-absorption efficiency; EPN, effective panicle number; GIR, grain nitrogen increase rate; GNPP, grain number per panicle; NUPE, nitrogen uptake efficiency; NUJTE, nitrogen utilization efficiency; PNUE, physiological nitrogen use efficiency; TRN, tillering response to nitrogen.

*Considered those QTLs that explained maximum phenotypic variations in the studied cultivars.

Table 4 List of NUE genes known in rice

Gene symbol (RAP-DB id)	Gene name	Transgenic nature	Subspecies	N-responsive/NUE phenotype	Direct or indirect role in NUE	Reference
N transport						
OsNRT2.3b (Os01g0704100)	High-affinity nitrate transporter 2.3	EMS mutant of OsNRT2.3b overexpression line	Japonica	EMS mutant had reduced growth time with improved nitrogen translocation efficiency, physiological and NUE	Direct	Chen et al. (2022)
OsAMT1;1 (Os04g0509600), OsAMT1;2 (Os02g0620600), OsAMT1;3 (Os02g0620500)	Ammonium transporter-1;1, and 1;2 and 1;3	Knockout triple mutant	Japonica	Decreased panicle number per plant and grain yield in the mutants.	Indirect	Konishi and Ma (2021)
OsNRT1.1B (Os03g0235900)	Nitrate transporter 1	Allelic variation	Indica vs Japonica	DNR1 ^{indica} allele promoted auxin-mediated grain yield and NUE in Indica.	Direct	Zhang et al. (2021)
OsNAR2.1 (Os02g0595900) and OsNRT2.3a (Os01g0704100)	Activator for NRT2 (OsNAR2.1) and high-affinity nitrate transporter 2.3 (OsNRT2.3a)	Co-overexpression	Japonica	Co-overexpression of both genes increased rice yield and NUE	Direct	Chen et al. (2020a)
OsAMT1;2 (Os02g0620600) and OsGOGAT1 (Os01g0681900)	Ammonium transporter-1;2 (OsAMT1;2) and NADH-glutamate synthase 1 (OsGOGAT1)	Activation tagging double mutant	Japonica	Concurrent activation of both the genes enhanced grain yield and NUE	Direct	Lee et al. (2020a)

OsLHT1 (Os08g0127100)	Lysine-histidine-like transporter 1	Knockout mutant	Japonica	Mutant showed decreased N uptake efficiency and physiological utilization efficiency	Direct	Guo et al. (2020)
NC4N	NC4N is a chimeric nitrate transporter, in which 2nd to 5th TMDs were replaced by the corresponding region of CHL1 transporter	NRT1.7p::NC4N::3	Japonica	Hyperactive chimeric nitrate transporter increased grain yield	Direct	Chen et al. (2020b)
OsAAP1 (Os07g0134000)	Amino acid permease 6	Overexpression and knockout (CRISPR)	Japonica	Overexpression increased Nue	Indirect	Ji et al. (2020)
OsDUR3 (Os10g0580400)	High-affinity urea transporter	Loss of function mutant	Japonica	Downregulation reduced yield	Indirect	Beier et al. (2019)
OsNRT1.1A/ OsNPF6.3 (Os08g0155400)	Nitrate transporter 1.1A	Overexpression and knockdown	Japonica	Overexpression increased Nue	Direct	Wang et al. (2018b)
OsNPF7.7 (Os10g0579600)	Peptide transporter 7.7 (both splice variants)	Overexpression	Japonica	Overexpression increased Nue	Direct	Huang et al. (2018)
OsAAP3 (Os06g0556000)	Amino acid permease 3	Overexpression and downregulation (CRISPR/Cas9)	Japonica	Downregulation enhanced Nue	Direct	Lu et al. (2018)
OsNAR2.1 (Os02g0595900)	Activator for NRT2	Overexpression	Japonica	Overexpression increased Nue	Direct	Chen et al. (2017)

(Continued)

Table 4 (Continued)

Gene symbol (RAP-DB Id)	Gene name	Transgenic nature	Subspecies	N-responsive/NUE phenotype	Direct or indirect role in NUE	Reference
OsPTR6/OsNPF7.3 (Os04g0597800)	Protein transporter 6/peptide transporter family 7.3	Overexpression and knockdown	Japonica	Overexpression increased NUE	Direct	Fang et al. (2017)
OsNRT2.1 (Os02g0112100)	High-affinity nitrate transporter 2.1	Overexpression (pOsNAR2.1:OsNRT2.1)	Japonica	Overexpression increased NUE	Direct	Chen et al. (2016)
OsNRT2.3b (Os01g0704100)	High-affinity nitrate transporter 2.3	Overexpression	Japonica	Overexpression increased NUE	Indirect	Fan et al. (2016)
OsAMT1.3 (Os02g0620600)	Ammonium transporter 1-3	Overexpression	Japonica	Overexpression decreased yield at high N	Indirect	Bao et al. (2015)
OsPTR6/OsNPF7.3 (Os04g0597800)	Protein transporter 6/peptide transporter family 7.3	Overexpression	Japonica	Overexpression decreased NUE	Direct	Fan et al. (2014)
OsAMT1;1 (Os04g0509600)	Ammonium transporter-1;1	Overexpression	Japonica	Overexpression increased NUE	Direct	Ranathunge et al. (2014)
OsPTR9 (Os06g0706400)	Protein transporter 9	Overexpression and knockdown mutant	Japonica	Overexpression increased NUE and yield	Direct	Fang et al. (2013)
OsNPF7.9 (Os02g0689900)	Nitrate transporter 1/peptide transporter Family protein 7.9	Loss of function mutant	Japonica	Mutant showed decreased grain yield and NUE	Direct	Guan et al. (2022)
N assimilation						
GS1;3 (Os03g0712800)	Cytosolic glutamine synthetase	Knockout mutant	Japonica	Observed decrease in agronomic traits	Direct	Fujita et al. (2022)
HvAlaAT	Alanine aminotransferase 1	Overexpression	Japonica	Overexpression increased grain yield and NUE	Direct	Tiong et al. (2021)

TaGS1	Glutamine synthetase 1	Overexpression	Japonica	Overexpression increased grain yield and NUE	Direct	Wu et al. (2021)
OsGS1;2 (Os03g0223400) and OsGS2 (Os04g0659100)	Glutamine synthetase	Overexpression and RNAi lines	Japonica	Overexpression increased the tiller number	Direct	Wang et al. (2020b)
OsASN1 (Os03g0291500)	Asparagine synthetase 1	Overexpression and knockout mutants	Japonica	Overexpression increased grain yield, Pn and protein content	Indirect	Lee et al. (2020b)
OsNR2 (Os08g0468700)	Nitrate reductase 2	Allelic variation	Indica vs Japonica	Increased effective tiller number, grain yield and NUE in Indica.	Direct	Gao et al. (2019)
OsGS1;2 (Os03g0223400)	Cytosolic glutamine synthetase 1;2	Overexpression	Japonica	Overexpression increased utilization NUE	Direct	Brauer et al. (2011)
CsAlaAT2	Alanine aminotransferase	Overexpression	Japonica	Overexpression increased NUE	Direct	Sisharmini et al. (2019)
HvAlaAT	Alanine aminotransferase	Overexpression	Japonica	Overexpression increased NUE	Direct	Shrawat et al. (2008)
NADH-GOGAT (Os01g0681900)	NADH-dependent glutamate synthase	Overexpression	Indica	Overexpression increased N utilization and grain filling	Indirect	Yamaya et al. (2002)
Transcriptional regulators						
OsNhd1 (Os08g0157600)	N-mediated heading date-1	Knockout mutant	Japonica	Mutant showed higher N uptake efficiency (NUpE)	Direct	Li et al. (2022)

(Continued)

Table 4 (Continued)

Gene symbol (RAP-DB id)	Gene name	Transgenic nature	Subspecies	N-responsive/NUE phenotype	Direct or indirect role in NUE	Reference
Ghd7 (Os07g0261200)-ARE1 (Os08g0224300)	Heading date 7 (Ghd7) and abnormal cytokinin response 1-1 repressor1 (ARE1)	Allelic variation	Japonica	Increased grain yield and NUE under low-N conditions	Direct	Wang et al. (2021b)
OsNAC68 (Os01g0675800)	NAC domain-containing protein 014	Overexpression	Japonica	Overexpression increased grain yield and NUE	Direct	Xin et al. (2021)
OsMYB305 (Os01g0637800)	Myb transcription factor	overexpression	Japonica	Overexpression increased N uptake and tiller number under low N	Indirect	Wang et al. (2020a)
ARE1 (Os08g0224300)	Abnormal cytokinin response 1-1 repressor1 (ARE1)	Loss of function mutant (allele)	Japonica	Knockdown of ARE1 increased NUE.	Direct	Wang et al. (2018a)
OsTBP2.1 (Os03g0657000)	TATA-box binding protein	Overexpression and knockout mutant	Japonica	Overexpression increased nitrogen uptake and yield	Direct	Zhang et al. (2022a)
Calcium-mediated signalling						
OsNLP3 (Os01g0236700)	Nodule inception (NIN)-like protein 3	Overexpression	Japonica	Overexpression increased grain yield and NUE	Direct	Zhang et al. (2022b)
OsNLP4 (Os09g0549450)	Nodule inception (NIN)-like protein 4	Overexpression and knockout mutants	Japonica	Overexpression increased tiller number, grain yield and NUE	Direct	Yu et al. (2021)

OsNLP1 (Os03g0131100)	Nodule inception (NIN)-like protein 1	Overexpression and knockout mutant	Japonica	Overexpression enhanced grain yield and NUE	Direct	Alfath et al. (2020)
OscIPK2 (Os07g0678600)	Calcineurin B-like interacting protein kinase 2	Overexpression	Japonica	Overexpression reduced PE, AE and RE at normal N condition	Direct	Khan et al. (2019)
G-protein-mediated signalling						
LW5/D1/RGA1 (Os05g0333200)	Leaf width 5/G-protein alpha subunit	Knockout mutant	Indica	Mutant showed increased N uptake efficiency	Indirect	Zhu et al. (2020)
DEP1 (Os09g0441900)	Dense and erect panicle 1	Overexpression of dep1 allele	Japonica	Overexpression increased agronomic traits including NUE	Direct	Zhao et al. (2019)
OsSPL14 (Os08g0509600)	Squamosa promoter- binding-like protein 14	Knockout mutants	Japonica	Knockouts of OsSPL14 showed reduced plant height and tiller number	Indirect	Jiang et al. (2020); Srikanth et al. (2016)
OsSPL18 (Os09g0507100)	Squamosa promoter- binding-like protein 18	Overexpression and knockout mutants	Japonica	OsSPL18 binds to the promoter of DEP1 (NUE QTL) and knockout mutant showed increased tiller number and reduced panicle length and grain number	Indirect	Yuan et al. (2019)
Protein modification and degradation						
OsATG8b (Os04g0624000)	Autophagy- associated gene 8B	Overexpression and knockdown mutant	Japonica	Overexpression conferred higher N recycling efficiency to grains	Direct	Zhen et al. (2021)

(Continued)

Table 4 (Continued)

Gene symbol (RAP-DB Id)	Gene name	Transgenic nature	Subspecies	N-responsive/NUE phenotype	Direct or indirect role in NUE	Reference
OsSAE1a (Os11g0497000)	SUMO-activating enzyme subunit 1	Knockdown mutants	Japonica	Down-regulation reduced panicle length and seed setting rate	Indirect	Wang et al. (2021d)
OsATG8a (Os07g0512200)	Autophagy-associated gene 8a	Overexpression	Japonica	Overexpression enhanced effective tiller numbers, NUpE and NUE	Direct	Yu et al. (2019)
OsATG8c (Os08g0191600)	Autophagy-associated gene 8c	Overexpression	Japonica	Overexpression increased yield, NUpE and NUE	Direct	Zhen et al. (2019)
OsPP2C9 (Os01g0846300)	Protein phosphatase 2C	Overexpression	Japonica	Overexpression increased NUE	Direct	Waqas et al. (2018)
OsBT (Os01g0908200)	BT1/BT2 ortholog	Loss of function mutant	Japonica	Loss of OsBT increased NUE	Direct	Araus et al. (2016)
Epigenetic regulation						
NGR5 (Os05g0389000)	Nitrogen-mediated tiller growth response 5	Loss of function mutant	Japonica	Mutant showed reduced tiller number, which was insensitive to change in N	Direct	Wu et al. (2020)
Stress						
OsLSD1.1 (Os08g0159500)	Lesion simulating disease 1	Knockout	Japonica	Mutant showed decrease in photosynthetic nitrogen use efficiency (PNUE)]	Direct	Xiang et al. (2021)

DREB1C (Os06g0127100)	Dehydration-responsive element-binding protein 1C	Overexpression lines and knockout mutants	Japonica	Overexpression lines showed increased yield and NUE due to enhanced nitrogen uptake and transport activity	Direct	Wei et al. (2022)
DST	Drought and salt tolerance	Loss of function mutant	Japonica	Mutant lines showed significant decrease in NUE	Direct	Han et al. (2022)
Miscellaneous						
PSF	Premature senescence of flag leaves	Gamma irradiation mutant	Indica	Increased N translocation efficiency in mutants	Indirect	Zakari et al. (2021)
OsENOD93-1 (Os06g0142350)	UDP-glucuronyl transferase early nodulin 93 gene 1	Overexpression	Japonica	Overexpression increased NUE	Direct	Bi et al. (2009)
OsPHR3 (Os02g0139000)	Phosphate starvation response 3	Overexpression	Japonica	Overexpression increased grain weight, grain yield and NUE	Direct	Sun et al. (2021)

HvAlaAT, *Hordeum vulgare* alanine aminotransferase; TaGS1, *Triticum aestivum* glutamine synthetase 1.

gene(s) responsible for observed phenotypes. N-responsive phenotypes have been used to identify the genes involved in NUE and recently discovered NUE phenotypes in rice (Sharma et al., 2021) would expedite the research on NUE. Traditional forward genetic approaches take longer time and, in many cases, lack the information on molecular basis and associated mechanisms responsible for observed phenotypes.

Reverse genetics begins with the known candidate gene and then generation of transgenic (loss or gain of function) plants to define the effect of manipulated gene(s) on the various traits/phenotypes. Advancements in genome sequencing information have provided lots of information about genes not only in the particular species but also in different species and germplasms. Therefore, reverse genetic approach permits tremendous possibility to develop and evaluate transgenic plants for NUE improvement under different N conditions. In the last two decades, the research on transgenic manipulation for NUE improvement has been focused on cereals including rice. We have covered the recent information on gene manipulation for NUE (Table 4) and also included information reported in earlier publications (Mandal et al., 2018; Raghuram and Sharma, 2019; Sinha et al., 2020; Kumari and Raghuram, 2020; Madan et al., 2022).

To identify the association of candidate genes with NUE, the most common targets for gene manipulations are N transporters (OsNPF7.9, OsNRT2.3b, OsAMT1;1, OsAMT1;2, OsAMT1;3), N assimilation enzymes (OsNR2, OsGS1;2, OsGS2, GS1;3, AlaAT) and genes involved in transcriptional regulations (OsTBP2.1, OsMYB305, OsNLP3, OsNLP4, OsNAC68, OsMYB305) as mentioned in Table 4 and similar family members in earlier publications (Mandal et al., 2018; Raghuram and Sharma, 2019; Sinha et al., 2020; Kumari and Raghuram, 2020; Madan et al., 2022). Identified NUE genes are associated with heading date (OsNhd1, Ghd7), autophagy pathway (OsATG8a, OsATG8b), epigenetic regulation (NGR5), stress (OsLSD1.1), calcium signalling (OsNLP1, 3 and 4) and other nutrient (OsPHR3) among others (Table 4).

4.4 Biological processes regulated by nitrogen use efficiency genes

The NUE genes mentioned in Table 3 were functionally categorized into various biological processes based on their validated biological functions known in the literature. The most prominent functional categories include N transport (37%), N assimilation (18%), transcriptional regulators (11%), protein modification and degradation (10%) and calcium-mediated signalling (7%) among others (Fig. 1). Details of biological processes are given later.

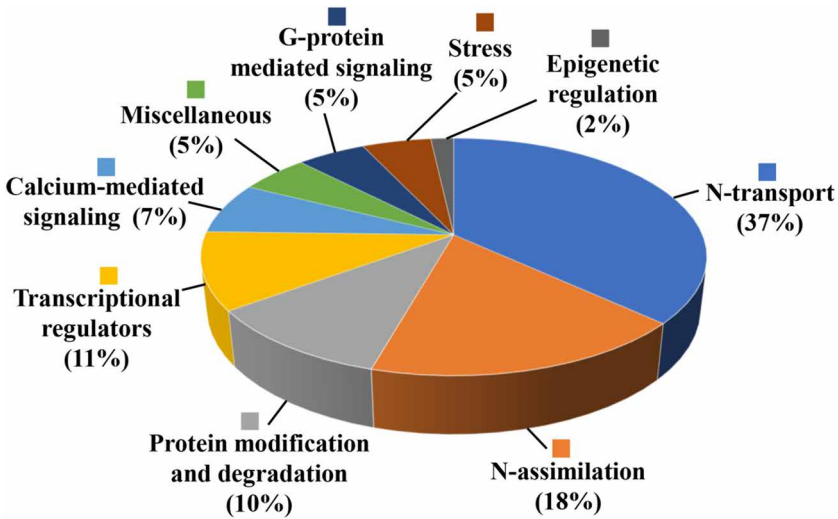


Figure 1 Pie diagram represents the functional categories of NUE genes known in rice. NUE genes were classified according to their function known in literature.

4.4.1 Nitrogen transport, nitrogen metabolism and nitrogen use efficiency

N uptake efficiency and subsequent utilization efficiency regulate NUE in plants. In rice, the majority of known NUE genes belong to the N transport (37%) and assimilation (18%) categories (Fig. 1). In rice, nitrate and ammonium are the two most common soil N forms, which are transported inside the cells using nitrate transporters (NRTs) and ammonium transporters (AMTs), respectively. Overexpression of NRTs such as OsNRT1;1A or AMTs such as OsAMT1;1 have been shown to improve the NUE, whereas their downregulation decreased NUE in rice (Table 4). This was not the case with OsAMT1.3, where its overexpression reduced yield at high N (Bao et al., 2015), which could be due to different transcriptional or translational regulation within the same gene families. Further, the triple mutants of OsAMTs (1;1, and 1;2 and 1;3) showed reduced NUE in rice (Konishi and Ma, 2021). Apart from nitrate and ammonium, amino acids present in the soil are acquired by the root through amino acid transporters (AATs). Amino acids are also generated through the reduction of nitrate via the GS-GOGAT pathway. In the context of remobilization, amino acids are considered the major source of N form and therefore play an important role in NUE. Downregulation of AATs such as amino acid permease 3 (Lu et al., 2018) and lysine-histidine-like transporter 1 (Guo et al., 2020) has been shown to reduce NUE in rice (Table 4). Considering the importance of AATs in N signalling, assimilation, metabolism and long-distance transport, limited

information is available for AATs in rice compared to Arabidopsis. Further, the coordinated role of NRTs, AMTs and AATs in NUE is yet to be established.

During N assimilation, nitrate reductase (NR) converts nitrate into nitrite, which is then converted into ammonium by nitrite reductase (NiR) in plants. Ammonium goes through a series of enzymatic reactions and is involved in the generation of different amino acids. The allelic variation of OsNR2 has demonstrated its role in NUE (Gao et al., 2019), whereas direct evidence supporting the role of NiR in rice NUE is yet to be established. Interestingly, manipulation of the promoter of NiR for the OsNLP4 transcription factor has improved yield at low N as compared to wild type in rice (Yu et al., 2021). Cytosolic and plastidic isoforms of glutamine synthetase (GS) are known in rice. Rice genome encodes three members of GS1 (GS1;1, GS1;2, and GS1;3) and overexpression of OsGS1;2 (Wang et al., 2020b) and GS1;3 (Fujita et al., 2022) improved NUE in rice (Table 4). The role of N-assimilation enzymes such as glutamate synthase (FD-GOGAT or NADH-GOGAT) in the context of other assimilatory enzymes and transporters is under progress (Li et al., 2022a) and needs deeper investigation for NUE improvement in rice.

4.4.2 Protein modification, degradation and nitrogen use efficiency

It has been established that post-translational modifications (PTMs) are involved in the regulation of protein stability, distribution and their function and therefore tightly control the biological responses in plants. PTMs play an important role in soil N uptake, assimilation and remobilization (Wang et al., 2021c). Phosphorylation is the most common PTM that occurs in plants. Phosphorylation of nitrate transporter (Ho et al., 2009) and ammonium transporter (Loque et al., 2007; Lanquar and Frommer, 2010) has been characterized, which suggests the importance of post-translational regulation to control the N uptake and translocation in plants. However, phosphorylation of urea transporter (DUR3) and phosphorylation-dependent urea uptake are not known in the plants.

The transporter NRT1.1 is known as a transceptor, i.e. it can act as a nitrate transporter and nitrate receptor in Arabidopsis. NRT1.1 works as a dual affinity transporter, i.e. it works as a low-affinity transporter under low nitrate availability, whereas phosphorylation of Thr101 amino acid by CIPK23 kinase converts NRT1.1 into a high-affinity nitrate transporter. Availability of high nitrate promotes dephosphorylation of Thr101 and NRT1.1 function as low-affinity nitrate transporter in Arabidopsis. Uptake of ammonium from soil is performed by ammonium transporters (AMTs) in plants. Time- and ammonium dose-dependent phosphorylation of T460 residue of Arabidopsis ammonium transporter AMT1.1 was observed (Loque et al., 2007). CIPK23 has been shown

to phosphorylate the T460 residue of AMT1.1 in Arabidopsis but the loss of function of the *ckp23* mutant provided evidence that additional kinases are involved in the phosphorylation of T460 residue in Arabidopsis (Straub et al., 2017). Addition of ammonium supply promoted T452 (similar to the T460 in Arabidopsis AMT1.1) phosphorylation in OsAMT1.2 by OsACTPK1 kinase in rice (Beier et al., 2018). More experimental pieces of evidence are needed to decode the transceptor function as in the case with Arabidopsis nitrate transporter NRT1.1. Based on the transceptor function of bacterial and fungal AMTs (Lanquar and Frommer, 2010), it was hypothesized that Arabidopsis AMTs may function as transceptor in plants. However, this hypothesis needs to be validated in plants.

N remobilization includes the transport of mainly nitrate and amino acids and therefore contributes an important step towards NUE improvement. Nitrogen limitation adaptation gene encodes a RING E3 ligase, which interacts and ubiquitinates with nitrate transporter AtNRT1.7 for its degradation via 26S proteasome pathway in Arabidopsis (Liu et al., 2017a). During N deficiency, ubiquitination controls the source-to-sink remobilization of leaf nitrate by regulating the protein abundance of AtNRT1.7 in Arabidopsis (Liu et al., 2017). Though the information is available on genes, proteins and enzymes involved in the ubiquitin-proteasome pathway, their role in rice NUE is not established.

Autophagy, an important component in the N remobilization, is an intracellular degradation process, which maintains cellular homeostasis under nutrient starvation condition by providing nutrients through the removal of damaged or aged proteins, or organelles in plants. It has been shown that overexpression of OsATG8a (Yu et al., 2019); OsATG8c (Zhen et al., 2019) increased NUE in rice (Table 4). These observations suggest that protein modifications have the unique potential to improve NUE and therefore we must explore these areas and identify candidate genes for improvement of NUE in rice.

4.4.3 Transcriptional regulators and nitrogen use efficiency

Transcription factors (TFs) bind to the cis-regulatory elements to regulate the expression of genes to control biological responses in plants. A transcriptional regulatory network (TRN) is formed by TFs and their interactions with target genes, and nitrate-regulated TRN has broadened molecular aspects of N transport and metabolism in Arabidopsis (Gaudinier et al., 2018). Despite the fact that such a comprehensive TRN is not known in crops, orthologs-based predicted N-responsive TRN revealed many TFs and their targets involved in N transport and metabolism in rice (Pathak et al., 2020; Sharma et al., 2022). Many TFs from various gene families have been characterized for their role in N response, but their role in NUE is limited in rice. For example, nitrate-regulated

1 (ANR1), a MADS-box TF, has been shown to regulate nitrate-dependent lateral root development in *Arabidopsis* (Zhang and Forde, 1998), whereas OsMADS25 regulates the nitrate-dependent architecture of the rice root system (Zhang et al., 2018). These TF family members have yet to be characterized for their role in the improvement of NUE in rice. Among TFs, NHD1, ARE1 and NAC68 are well-known TFs that regulate NUE in rice (Table 4).

4.4.4 Calcium signalling and nitrogen use efficiency

Nitrate-regulated primary responses include activation of many genes involved in myriads of pathways including calcium (Ca^{2+}) signalling in plant (Liu et al., 2020). Ca^{2+} -mediated signalling is one of the most evolutionarily conserved signalling, involved in the regulation of various physiological, stress, and transport processes in plants. Nitrate has been shown to induce Ca^{2+} ions level in cytosol and nucleus; however, such induction was not observed in nitrate transporter NRT1.1 mutants, suggesting nitrate-specific Ca^{2+} induction in *Arabidopsis* (Riveras et al., 2015). Calcium signalling involves Ca^{2+} ions sensor proteins such as calcineurin-B-like proteins (CBL) and calcium-dependent protein kinases (CPKs), which interact with and activate CBL-interacting protein kinases (CIPK) for the phosphorylation of target proteins for downstream signalling (Liu et al., 2021a).

Under low N conditions, CBL proteins (CBL1 and CBL9) interact with CIPK23 and form associated complexes (CBL1-CIPK23 and CBL9-CIPK23), which convert low-affinity nitrate transporter NPF6.3/NRT1.1 into high affinity after phosphorylation (Ho et al., 2009). The ABI2 protein, a specific protein phosphatase 2C, dephosphorylate NPF6.3/NRT1.1 transporter to antagonize CBL1/CBL9-CIPK23 complex phosphorylation responses in *Arabidopsis* (Leran et al., 2015). Other Ca^{2+} sensor proteins CPKs (CPK10, CPK30 and CPK32) are known to phosphorylate TF NIN-like protein-7 in the nucleus, which regulate a large set of genes to implement nitrate response in rice (Liu et al., 2017b). There are many Ca^{2+} signalling genes have been characterized for their N responses in *Arabidopsis* and rice, but only NLPs and CIPK2 have been shown to regulate NUE in rice (Table 4). The role of other core components of Ca^{2+} signalling in the regulation of rice NUE is yet to be established.

4.4.5 G-protein signalling and nitrogen use efficiency

Heterotrimeric G-proteins, which consist of three different subunits (α , β , and γ), have been implicated in the regulation of plant growth, development, stress and nutrient responses in rice (Cui et al., 2020; Pandey, 2019). Our earlier molecular studies, including genome-wide N responses, have demonstrated the role of G-proteins in the regulation of N responses in *Arabidopsis* (Chakraborty et al.,

2015a, 2019), maize (Raghuram et al., 1999) and rice (Ali et al., 2007; Pathak et al., 2021; Prasanna et al., 2023). To identify the genetic loci responsible for NUE, QTL mapping experiments were performed using rice genotypes with significant variation in the N-responsive traits, which led to the identification of a major QTL (qNGR9) for NUE in rice (Sun et al., 2014). Fine mapping of qNGR9 and genetic complementation experiments revealed a previously identified gene known as Dense and Erect Panicles 1 (DEP1). DEP1 is a G-protein gamma subunit, and the gain of function of *dep1* allele improved NUE in rice (Sun et al., 2014). Members of the gene family 'squamosa promoter binding protein-like' (OsSPL) have been shown to regulate growth and development in rice. For example, OsSPL14 regulates ideal plant architecture and it was differentially regulated under low N conditions (Srikanth et al., 2016). The OsSPL18 knockout mutant showed reduced panicle length and grain number while the number of tillers increased (Yuan et al., 2019). Further, OsSPL18 also binds to the promoter of DEP1, which controls NUE in rice. This clearly suggests their potential involvement in the regulation of NUE in rice.

In another study, EMS (ethyl methanesulfonate) mutants of indica rice were analysed, and the leaf width 5 (*lw5*) mutants were identified, which had broad width leaves and small grains (Zhu et al., 2020). Map-based cloning revealed the base substitution mutation in the G α subunit (RGA1) leading to truncated RGA1 proteins in the mutants. This mutant showed improved photosynthetic and N utilization efficiency as compared to the wild-type plants (Zhu et al., 2020). Advancements in G-protein signalling research have expanded the signalling components in plants. The Arabidopsis G-protein interactome (Kloppfleisch et al., 2011) and orthologs-based predicted rice G-protein interactome (Pathak et al., 2021) revealed many known and potential candidates associated with N responses/NUE in rice. As compared to the available information and identification of NUE QTL, this area is underexplored and needs much deeper investigation for the improvement of NUE.

4.4.6 Epigenetic regulation, stress and nitrogen use efficiency

Pie diagram shows that N transport and N assimilation are relatively more explored areas for NUE improvement, whereas biological processes such as 'Epigenetic regulation' and 'Stress' need much deeper investigation in the context of NUE (Fig. 1). Apart from transcriptional and post-transcriptional regulation, that epigenetic regulation also affects the expression of genes through different processes of DNA methylation and histone modifications in plants (Liang et al., 2020). It is known that N affects the DNA methylation status as well as histone modification dynamics in plants (Kou et al., 2011; Secco et al., 2017). The NR gene was methylated by chromomethylase 3, which was regulated by ammonium in Arabidopsis (Kim et al., 2015). It has been shown that histone

Table 5 List of intergenic markers developed for NUE genes in rice

Gene symbol (Id)	Gene name	Molecular marker name	Molecular marker type	Reference
SBM1 (LOC_Os01g65120)	Peptide transporter	SBM1 5U ID and SBM1 E3S	Indel and CAPS	Xu et al. (2021)
OsTCP19 (LOC_Os06g12230)	Teosinte branched/cycloidea/proliferating cell factors 19	TCP19 5U ID	Indel	Liu et al. (2021b)
OsNLP4 (LOC_Os09g37710)	Nodule inception (NIN)-like protein 4	NLP4 5U ID	Indel	Yu et al. (2021)
DNR1 (LOC_Os01g08270)	Aminotransferase	DNR1 14 ID	Indel	Zhang et al. (2021)
NGR5 (LOC_Os05g32270)	Nitrogen-mediated tiller growth response 5	NGR5 5U ID and NGR5 16 S	Indel and PARMS	Wu et al. (2020)
MYB61 (LOC_Os01g18240)	Myb transcription factor	MYB61 3U ID	Indel	Gao et al. (2020)
OsNAC42 (LOC_Os09g32040)	NAC domain-containing protein 042	NAC42 3U S	dCAPS	Tang et al. (2019)
OsNR2 (LOC_Os02g53130)	Nitrate reductase 2	NR2 E4 ID	Indel	Gao et al. (2019)
OsNPF6.1 (LOC_Os01g01360)	Nitrate transporter 1	NPF6.1 5U ID	Indel	Tang et al. (2019)
NGR2/GRF4 (LOC_Os02g47280)	Growth-regulating factor 4	NGR2 5U S and NGR2 13 S	CAPS and PARMS	Li et al. (2018)
ARE1 (LOC_Os08g12780)	Abnormal cytokinin response 1-1 repressor1	ARE1 5U ID and ARE1 5U S	Indel and CAPS	Wang et al. (2018a)
NRT1.1B/OsNPF6.5 (LOC_Os10g40600)	Nitrate transporter 1	NRT1.1B 11 ID	Indel	Hu et al. (2015)
TOND1 (LOC_Os12g43440)	Tolerance of nitrogen deficiency 1	TOND1 5U ID	Indel	Zhang et al. (2015)
DEP1 (LOC_Os09g26999)	Dense and erect panicle 1	DEP1 E5 ID	Indel	Sun et al. (2014)

This adapted table was created based on the information provided in earlier publication (Li et al., 2022c).

H3 lysine 27 trimethylation (H3K27me3) regulates N-mediated tillering in rice (Wu et al., 2020). To promote N-mediated tillering, branching-inhibitory genes are inhibited after H3K27me3 modification by polycomb repressive complex 2, which was recruited by N-mediated tiller growth response 5 (NGR5) TF in rice (Wu et al., 2020). Despite available epigenetic regulation information, only NGR5 has been shown to regulate NUE in rice (Table 4). This clearly showed that the identification and manipulation of genes associated with epigenetic regulation may improve NUE in rice.

Crosstalk of N and stress signalling has been a relatively underexplored research area for crop improvement (Jangam and Raghuram, 2015). N and stress responses showed commonality in terms of physiological, biochemical and molecular changes in plants. For example, the roots are the primary organ to sense soil N fluctuation and drought condition. Photosynthesis, chlorophyll contents, etc. are affected under both conditions. Further, mapping of nitrate- or urea-responsive differentially expressed genes onto various biological pathways and processes revealed their involvement in abiotic and biotic stresses in rice (Pathak et al., 2020; Sharma et al., 2022; Mandal et al., 2022; Sharma et al., 2023). Few stress-associated genes have been characterized for their role in NUE (Table 4). For example, a recent study showed that the drought and salt tolerance (DST) TF regulates NUE by controlling the N assimilation gene *OsNR1.2* in rice (Han et al., 2022). The loss of function of *dst* mutant showed reduced *OsNR1.2* function and accordingly a significant reduction in NUE (Han et al., 2022). In another study, the screening of 118 potential TFs identified from different transcriptomic and metabolomics studies led to the identification of Dehydration-Responsive Element-Binding Protein 1C (*OsDREB1C*), which improved photosynthesis and NUE in rice (Wei et al., 2022). *OsDREB1C* is an AP2/ERF family TF, and its overexpression increased yield by up to 68%, including NUE in rice (Wei et al., 2022). These findings clearly show that unravelling the mechanisms controlling both N and stress responses and manipulating the identified candidate genes would provide tolerance to the plants and reduce the consumption of N fertilizers, contributing to sustainable agriculture.

4.5 Genetic and genomic strategies for manipulation of nitrogen use efficiency genes

Overexpression or mutation (knockdown or knockout) of these genes either increased or decreased the NUE in rice (Table 4). Interestingly, in few cases, two genes were manipulated to improve the NUE in rice (Table 4). For example, Chen et al. (2020a) demonstrated that transgenic plants co-expressing nitrate transporter (*OsNRT2.3a*) along with its interactor (*OsNAR2.1*) had increased NUE as compared to those single-gene transgenic rice plants overexpressing

either OsNRT2.3a or OsNAR2.1 in rice. In this case, both genes are involved in the same transport pathway. In another example, two genes involved in two different pathways viz. ammonium transport (OsAMT1;2) and N-assimilation pathways (OsGOGAT1) were co-activated in rice using T-DNA activation tagging method (Lee et al., 2020a). Rice plants with double activation of ammonium transporter (OsAMT1;2) and NADH-glutamate synthase 1 (OsGOGAT1) showed improved grain yield under N-limiting condition. This clearly suggests that to deal with complex nature of NUE, we must simultaneously manipulate multiple targeted genes involved in same or different pathways for improved NUE in rice.

Allelic variations within subspecies and related germplasms have provided the natural resources to identify the target loci/genes responsible for the phenotypic variation including NUE (Table 2). A study conducted by Gao et al. (2019) showed that allelic variation in nitrate reductase 2 (OsNR2), a gene involved in N-assimilation pathway, is responsible for high NUE in Indica rice as compared to Japonica subspecies. They showed that Trp₇₇₉ substitution in OsNR2 enzyme reduced its activity in Japonica as compared to Indica subspecies. In another study, a natural allelic variation was identified in nitrate transporter, which improved NUE by regulating auxin biosynthesis in rice (Zhang et al., 2021). An interesting work was performed by Chen et al. (2020a), where they first improved the NUE by overexpressing nitrate transporter (OsNRT2.3b) in rice. Next, they mutagenized the transgenic rice with EMS and isolated a transgenic plant having short duration with similar NUE. This study shows the integration of two methods, i.e. transgenic manipulation of a gene and screening of genetic variation (EMS mutagenesis) to develop a plant with multiple beneficial traits.

Improvement of NUE using chimeric protein is a unique methodology demonstrated by Chen et al. (2020b). They developed a chimeric nitrate transporter by replacing the transmembrane domain (TMD) region between second and fifth with CHL1 transporter. They observed increased NUE in Arabidopsis and grain yield in rice. This study provides an opportunity to develop synthetic chimeric protein (genotype-specific/inhibitory/activating domain) for NUE improvement. Consideration of synthetic promoter associated with stress and or nutrient responses would add another level of control to fine-tune the spatio-temporal regulation of chimeric protein for NUE improvement in rice.

4.6 Molecular markers for nitrogen use efficiency genes in rice

Molecular markers help in marker-assisted selection, which has been shown to improve the efficiency of rice cultivars. Research on NUE revealed many genes involved in NUE in rice. Recently, Li et al. (2022c) have developed the 18 intragenic

molecular markers to track the coinheritance of 14 NUE genes studied in rice (Table 5). These are NRT1.1B, OsDEP1, OsNR2 and OsDNR1 among others, which are well established for their role in NUE in rice (Table 5). To develop the markers, 2 kb upstream region from start codon and 1 kb downstream region from stop codon were considered. While developing these intragenic markers, they observed structural variations in the 5' UTR, which help to define genetic diversity across different germplasms. Identification of molecular markers associated with NUE is a necessary step for the wider application of discovered genes and to expedite the breeding programmes for NUE improvement.

5 Conclusion and future prospects

Improvement of NUE is one of the most important steps for sustainable agriculture, as well as for global sustainable N management. As a crop that feeds half the world and is one of the highest consumers of N fertilizers, rice is an important target crop for NUE. For improved NUE, either higher yields must be achieved using current N fertilizer inputs, or, even better, similar yields must be achieved using lower N inputs. Many studies have been performed to identify the molecular targets and associated signalling networks involved in rice NUE. Transgenic manipulation of selected molecular targets has improved yield and NUE in rice (Tables 3 and 4). Biological pathways primarily associated with manipulated NUE genes were N uptake, assimilation, translocation and remobilization. In the context of signalling events, N affects calcium-mediated signalling, hormone-dependent regulation, autophagy pathways and epigenetic regulation in rice (Table 4). Recent studies using other gene family members, such as calcium signalling associated NLP gene family, were characterized for NUE in rice. Further, NUE regulation through OsNGR5 opens an underexplored area of N and epigenetic regulation in plants.

During early studies, single-gene-based transgenic plants were used to understand the N response and NUE, but it appeared that this method does not provide complete information to improve NUE because of its complex nature. As per improved knowledge, manipulation of two or more genes involved in the same pathways has yielded better results in terms of NUE. For example, co-activation of nitrate transporter and its interactors provided increased NUE as compared to their single-gene transgenics (Chen et al., 2020). Since genotype and environment interaction affects NUE, we must also consider temporal and spatial gene regulatory networks controlling NUE in rice. Another underexplored area is gene splicing events and NUE. Alternative splicing of a gene produces different transcript variants, and it is possible that one or all splice variants may contribute to a particular trait or response. Liu et al. (2022c) have recently shown that glutamine synthase (*OsGS1;1*) has two splice variants

viz. *OsGS1;1a* and *OsGS1;1b* and the high *OsGS1;1b* activity is associated with improved NUE in rice. This will help to shortlist/identify the specific transcript variants that actually contribute to NUE.

The development of 'omics' approaches enabled us to identify the temporal and spatial gene regulatory networks controlled by N in rice. However, such studies are limited in terms of comprehensive tissues-, time- and N dose-specific transcriptional regulation in diverse cultivars and not available for translational or post-translational regulations and metabolic regulation with multiple rice cultivars (genotypes). For reproducible and reliable conclusions of the 'omics' data, we must generate N-dose dependent, comprehensive temporal and spatial data sets in many rice genotypes contrasting for NUE in field condition. This will help to understand the genotype and environment interaction, and accordingly manipulation of candidate genes for their temporal and/or tissue-specific roles for NUE.

The development of rice cultivars with a synthetic control system for gene expression would be one of the most challenging tasks in the near future. We can modulate the function of specific gene(s) according to the soil N condition, environment and developmental stages of plants by spraying/supplying exogenous activator. This will also help to engineer the plant for dual or multiple regulatory system, i.e. regulation at transport for efficient N uptake from soil and regulation at the reproductive stage for improved yield at low N.

The number of QTLs and associated genes for NUE are increasing with time, but very few of them are used in the breeding programmes as the molecular markers for many QTLs and NUE genes validated in field condition are not available. In this context, Li et al. (2022c) have developed the molecular markers for validated 14 NUE genes in rice cultivars. In addition, CRISPR/Cas9 is the most important genome editing tool for precision breeding in plants. This method has the ability to generate transgene-free rice plants. After the identification of elite rice varieties with improved NUE and the responsible gene(s), CRISPR/Cas9 genome editing tool can be used to manipulate the genome to generate many novel germplasms, which contain homologous NUE gene(s) identified in elite rice varieties. This will help to expedite the breeding programmes for NUE improvement in rice.

6 Where to look for further information

Sutton et al. (2019), Raghuram et al. (2021), Sutton et al. (2021) and Raghuram et al. (2022a) provide detailed information on the scientific community, international collaborations, and the importance of global governance to regulate N policy and global climate change.

Liu et al. (2022a) and Hawkesford and Griffiths (2019) provide comprehensive information on the molecular and genetic aspects of N-related processes and NUE in crop plants.

Current progress related to N can be found on the following websites:

- International Nitrogen Management System (<https://www.inms.international/>).
- International Nitrogen Initiative (<https://initrogen.org/>).
- Global Partnership on Nutrient Management (<http://www.nutrient-challenge.org/>).

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