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In silico Analysis of *OsNRT2.3* Reveals *OsAMT1.3*, *OsZIFL9*, *OsbZIP27*, and *OsIRT1* as Potential Drought-Related Genes During Nitrogen Use Efficiency in *Oryza sativa* L.

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ABSTRACT

Nitrate transporter (NRT) is responsible for the molecular mechanism of the root nitrate (NO_3^-) uptake system for plant development. Although several *NRT* genes are identified and characterised in plants, knowledge of the *NRT2* gene family and its nitrogen use efficiency (NUE) function in drought stress has remained elusive in rice. This study conducted an *in silico* analysis on 20 NRT2 family genes of rice, wheat, soybean, barley, maize, and papaya. Phylogenetic and motifs analysis clustered genes encoding NRT2 proteins into four monophyletic groups, and the motifs of *NRT2* genes were significantly conserved for the specific domain of NO_3^- transmembrane transporter. Interestingly, co-expression analysis revealed that potential drought-related genes were expressed similarly to the functional NUE gene, *OsNRT2.3*. Furthermore, half of the co-expressed genes were enriched in nitrogen use efficiency (NUE)-related processes, such as transport, stress, macromolecule metabolic pathways, and transcription regulation. Expression pattern analysis of *OsNRT2.3* and its co-expressed genes in tissue-specific and nitrogen (N) response led to the discovery of *OsAMT1.3*, *OsZIFL9*, *OsbZIP27*, and *OsIRT1* as four strong candidates to participate in drought stress during NO_3^- uptake system. The co-expression of iron (Fe) uptake genes, *OsZIFL9* and *OsIRT1*, with *OsNRT2.3* also suggested a possible interaction of Fe and

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nitrogen (N) during an increasing amount of Fe, which led to the acidification of rice apoplasts during water deficiency. Together, this study will provide a valuable resource for potential candidate genes that can further investigate their molecular response to drought during NUE.

Keywords: Drought stress, *in silico*, nitrogen use efficiency, *NRT2.3*, *Oryza sativa* L.

INTRODUCTION

Rice (*Oryza sativa* L.) is a major staple food consumed by the world's population, particularly in Asian countries. According to the Food and Agriculture Organization of the United Nations (FAO) (2017), the global population is expected to reach 9 billion by 2050, with food supplies increasing by 70% to 100%. Therefore, rice production must be significantly increased to meet the outburst of the world population. As one of the world's largest consumers, Malaysia has been importing 30% to 40% of its rice consumption annually for the last 30 years and will continue to be a net rice importer in the future (Khazanah Research Institute, 2019). To ensure the country's demand in the future, Malaysia must alleviate the production of high-quality rice by adhering to good agricultural practices. However, farmers face frequent diseases and pests, resulting in significant economic loss. Therefore, nutrient management has emerged as a critical, cost-effective strategy for increasing crop productivity in intensive agricultural practices worldwide, considering the abovementioned points.

Nitrogen (N) is an essential macro element for plant growth and development. N availability is a major limiting factor in rice crop growth and yield among the macroelements. Nitrogen is required in large quantities compared to other nutrients (Djaman et al., 2018). Thus, proper N fertiliser applications are crucial to enhance grain yield and grain quality in rice farming. Unfortunately, excessive use of N fertiliser in rice farming causes irreparable damage to soil structure, mineral cycles, soil microbial flora, and plants (K. Wu et al., 2021).

NUE, in general, consists of two key components: N uptake efficiency (NUpE), which is the efficiency of absorption of supplied N, and N utilisation efficiency (NUtE), which is the efficiency of assimilation and remobilisation of plant N in producing grain. NUE is inherently complex, owing to the interaction of multiple genes with environmental factors. N was primarily obtained by the plant in the form of nitrate (NO_3^-) or ammonium (NH_4^+) (Ranade-Malvi, 2011; Xu et al., 2012). The anionic form of NO_3^- is easily soluble in water and easily mobilised in soil (Jin et al., 2015). NO_3^- is absorbed through roots and transported to all parts of the plant via NO_3^- transporters or by producing amino acids through an assimilation process before being redistributed (Xu et al., 2012). Plants developed two nitrate transport systems to regulate nitrate: a high-affinity NO_3^- transport system (HATS) and a low-affinity NO_3^- transport system (LATS) (Huang et al., 2018).

Nitrate transporters are divided into four prominent protein families, including nitrate transporter 1 (NRT1) or peptide transporter (PTR), also known as NPF, nitrate transporter 2 (NRT2), chloride channel (CLC), and slow anion channel (SLAC1/SLAH) (Y. Y. Wang et al., 2012). Many studies on the molecular mechanism of the root nitrate uptake system have revealed that nitrate transporter gene families, *NRT1* and *NRT2*, were involved in the LATS and HATS systems, respectively (Fenchel et al., 2012; Forde, 2000; Orsel et al., 2002). In *Arabidopsis*, *AtNRT2.1* was reported as crucial in root architecture response due to low nitrate accumulation during lateral root initiation (Little et al., 2005; Remans et al., 2006). However, most of the NRT2 family members cannot transport NO_3^- independently as a pair protein called nitrate transporter-activating protein 2 (NAR2) is required to play a role in nitrate assimilation. In rice, three *OsNRT2* members, including *OsNRT2.1*, *OsNRT2.2*, and *OsNRT2.3a*, need the paired protein, *OsNAR2.1*, to absorb nitrate, whereas *OsNRT2.3b* and *OsNRT2.4* can function independently (Wei et al., 2018). Interestingly, few studies were conducted on *OsNRT2.3*, despite its importance in regulating NO_3^- transport in the root system as the root architecture changes in response to N-deficiency, thereby improving the NUE.

Drought and N deficiency induce adaptative responses such as root elongation, cell-damaging ROS production, and photosynthesis reduction. For example, reactive oxygen species (ROS) levels were reported to elevate in certain root areas during N deficiency due to reduced photosynthesis caused by drought stress (Safavi-Rizi et al., 2021). Previous studies have reported that drought stress affects the expression of key genes or QTLs associated with nitrogen management in rice. For instance, *ARE1* is associated with high yield under N-deficiency (Q. Wang et al., 2018), *qGYLN7* and *qGYPP-4b* increase grain yield under low N conditions (H. H. Tong et al., 2011; Rao et al., 2018), and *qRL6.1* enhances root elongation under deficient NH_4^+ condition (Obara et al., 2010).

In this study, *in silico* analyses were conducted to gain insights into an exhaustive knowledge of the function of the high-affinity nitrate transporter gene 2.3 (*NRT2.3*) in *O. sativa*. The members of the NRT family in rice and selected plants, such as soybean, maize, papaya, *Arabidopsis*, wheat, and barley, were analysed by executing bioinformatics approaches on the publicly available genome and protein sequences. In particular, a common and distinct organisation of functional motifs was identified among the phylogenetic tree of the NRT family, depicting evolutionary relationships among them. Subsequently, a *cis*-element regulatory analysis was also examined to determine the involvement of *OsNRT2.3* as a stress-responsive protein during NUE. Finally, co-expression analysis was performed to decipher the potential function of *OsNRT2.3* with correlated genes in NUE processes and later observed their expression profiles using publicly available microarray data. Protein-protein interaction analysis suggests that most correlated *OsNRT2.3* genes may interact

under the NUE functionally. The findings may contribute to a better understanding of its role in NUE for the genetic improvement of agronomic crops.

MATERIALS AND METHODS

Retrieval of NRT2 Family Gene Sequences from Databases

The complete coding sequence and protein sequence of the high-affinity nitrate transporter 2, NRT2 family genes from rice (*Oryza sativa*) and selected species, such as soybean (*Glycine max*), maize (*Zea mays*), papaya (*Carica papaya*), and *Arabidopsis* (*Arabidopsis thaliana*), were obtained from Plaza v3.0 (https://bioinformatics.psb.ugent.be/plaza/) (Proost et al., 2015). Meanwhile, *NRT2* sequences of wheat (*Triticum aestivum*) were obtained from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov) (Sayers et al., 2021) and barley (*Hordeum vulgare*) from GrainGenes (https://wheat.pw.usda.gov) (Matthews et al., 2003). The pI/Mw tool (https://web.expasy.org/compute_pi/) was employed to calculate the molecular weight (Mw) and isoelectric point (pI) of the NRT2 encoding protein by setting the resolution set to average (Gasteiger et al., 2005).

Phylogenetic Analysis

The multiple sequence alignment and phylogenetic analyses were conducted to evaluate the evolutionary relationships of the NRT2 family in their respective monophyletic groups or clades. First, the NRT2 protein sequences of rice, wheat, soybean, barley, maize, and papaya were aligned at default settings to determine their consensus and conserved regions using the MUltiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm (https://www.ebi.ac.uk/Tools/msa/muscle/) (Edgar, 2004). Subsequently, the MUSCLE alignment file was used to conduct the phylogenetic analysis using Molecular Evolutionary Genetics Analysis (MEGA7) software (https://www.megasoftware.net/) (Kumar et al., 2016). Next, the Maximum Likelihood (ML) phylogenetic tree was generated using the bootstrap method of 1,000 replications and the substitution method of the Jones-Taylor-Thornton (JTT) model at uniform rates. The phylogenetic tree was then annotated using Interactive Tree Of Life (iTOL) (https://itol.embl.de) (Letunic & Bork, 2007).

Conserved Motif Analysis and Motif Function Prediction

The conserved motif searching for the protein sequence of *NRT2* was conducted using the Multiple Expectation Maximisation for Motif Elicitation (MEME 5.3.3) online tool (https://meme-suite.org/meme/tools/meme) (Bailey et al., 2009). The search parameters used to discover the motifs were as follows: number of motifs = 20; minimum width = 6; maximum width = 50; minimum sites = 2; and maximum sites = 600. The HyperText Markup Language (HTML) output file exported from MEME was used to manually illustrate the organisation of the motifs on the protein sequences using the Illustrator for Biological Sequences (IBS) online tool (http://ibs.biocuckoo.org)

(Liu et al., 2015). The 20 consensus motifs sequences in *NRT2* were then annotated for their putative function using the Conserved Domains Database (CDD) (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) (Marchler-Bauer et al., 2015), Pfam (http://pfam.xfam.org) (Mistry et al., 2021), and PROSITE (https://prosite.expasy.org) (Hulo et al., 2006).

Promoter Analysis

In this study, the 2.0 kb upstream genomic region from the transcription site of *OsNRT2.3* was extracted from PLAZA 3.0 Monocots (https://bioinformatics.psb.ugent.be/plaza/) to perform promoter analysis (Proost et al., 2015). The 2.0 kb upstream region of the promoter sequence was searched against PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) to investigate the putative role of the *cis*-element (Lescot et al., 2002).

Analysis of Gene Co-Expression Network, GO Annotation, and Expression Patterns

The Gene Co-expression Network Analysis (GCNA) was conducted by retrieving publicly available co-expression data from the Rice Expression Database (RED) (http://expression.ic4r.org/) (Xia et al., 2017). The *OsNRT2.3* identity (LOC_Os01g50820/ Os01g0704100) was used as a query to identify and obtain the *NRT2.3* co-expression network, with Pearson's *r*-value set at 0.85. The network was later downloaded and visualised in the Cytoscape software (version 3.7.1) for further observation (Shannon et al., 2003). The Gene Ontology (GO) annotation of the co-expressed genes was then conducted to discover their function using the AgriGO database (http://bioinfo.cau.edu.cn/agriGO/index.php) (Tian et al., 2017). The Fisher statistical test method < 0.05 was selected for complete GO analysis. For expression profile analysis of genes of interest, the ePlant database (http://bar.utoronto.ca/eplant/) (Waese et al., 2017) was employed to examine co-expressed genes manifesting similar expression profiles to *OsNRT2.3* at a tissue-specific level. Rice whole-genome Affymetrix GeneChip array data for transcript analysis of N response was obtained from NCBI Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) (Cao et al., 2012) under accession number GSE61370 (Coneva et al., 2014).

Protein-Protein Interaction Network Analysis

After identifying co-expressed genes to *OsNRT2.3*, the protein-protein interactions (PPIs) data from STRING (https://string-db.org) (Mering et al., 2003) were retrieved using Cytoscape StringApp plugin to construct the PPI network of *NRT2.3* protein. The PPIs with a confidence score of > 0.400 were retained and visualised in the Cytoscape software (Shannon et al., 2003). However, in this study, only evidence of known interactions was selected, such as from a curated database, and experimentally determined to supply strong evidence of PPI via validated physical interactions between

the proteins. The PPI between *OsNRT2.3* and co-expressed gene encoding protein will strongly support their critical role in NUE at the functional level.

RESULTS

Identification of NRT2 Family Genes from Selected Organisms

The present study identified 20 selected NRT2 proteins and gene sequences, including four *NRT2* in rice named *OsNRT2.1* to *OsNRT2.4*, followed by seven *Arabidopsis NRT2* (*AtNRT2.1* to *AtNRT2.7*), four maize *NRT2* (*ZmNRT2.1* to *ZmNRT2.3*, and *ZmNRT2.5*), and two barley *NRT2* (*HvNRT2.5* and *HvNRT2.6*). Single *NRT2* was identified in soybean (*GmNRT2*), papaya (*CpNRT2*), and wheat (*TaNRT2.1*). The size of the proteins encoded by *NRT2* genes varies substantially. The length of NRT2 family proteins ranged from 485 to 542 amino acid residues. The shortest NRT2 protein is OsNRT2.4, 485 amino acids, and the most extended NRT2 protein is AtNRT2.6, 542 amino acids. The Expasy analysis demonstrated a large variation in isoelectric point (pI) values ranging from 5.40 to 9.21 and molecular weights ranging from 50.199 to 58.637 kDa. The information on the NRT2 proteins is reported in Table 1.

Table 1
The list of high-affinity nitrate transporter 2 (NRT 2) protein families

Protein ID	Gene name	Species	ORF length (bp)	Protein		
				Length (aa)	MW (kDa)	pI
OS02G02170	*OsNRT2.1*	*Oryza sativa*	1,602	533	57.230	8.43
OS02G02190	*OsNRT2.2*	*Oryza sativa*	1,602	533	57.230	8.43
OS01G50820	*OsNRT2.3*	*Oryza sativa*	1,551	516	55.407	8.99
OS01G36720	*OsNRT2.4*	*Oryza sativa*	1,452	485	50.199	8.37
ABG20828	*HvNRT2.5*	*Hordeum vulgare*	1,545	514	55.376	8.98
ABG20829	*HvNRT2.6*	*Hordeum vulgare*	1,524	507	54.673	8.39
AAG01172	*TaNRT2.1*	*Triticum aestivum*	1,796	507	54.708	8.39
ZM04G41480	*ZmNRT2.1*	*Zea mays*	1,755	524	56.675	8.20
ZM04G41500	*ZmNRT2.2*	*Zea mays*	1,855	524	56.644	8.20
ZM05G17230	*ZmNRT2.3*	*Zea mays*	2,001	522	56.099	8.35
ZM08G26310	*ZmNRT2.5*	*Zea mays*	2,094	520	55.775	8.94
AT1G08090	*AtNRT2.1*	*Arabidopsis thaliana*	2,111	530	57.709	8.85
AT1G08100	*AtNRT2.2*	*Arabidopsis thaliana*	1,709	522	56.615	8.72
AT5G60780	*AtNRT2.3*	*Arabidopsis thaliana*	1,922	539	58.230	8.96
AT5G60770	*AtNRT2.4*	*Arabidopsis thaliana*	1,716	527	57.768	9.04
AT1G12940	*AtNRT2.5*	*Arabidopsis thaliana*	1,852	502	54.261	8.97
AT3G45060	*AtNRT2.6*	*Arabidopsis thaliana*	1,912	542	58.637	8.84
AT5G14570	*AtNRT2.7*	*Arabidopsis thaliana*	1,917	493	52.677	5.40
GM08G39140	*GmNRT2*	*Glycine max*	2,099	508	55.259	9.21
CP00056G00130	*CpNRT2*	*Carica papaya*	1,638	534	57.986	9.12

Note. ORF = Open reading frame ; MW = Molecular weight ; pI = Isoelectric point

Phylogenetic Classification of NRT2 Family Genes

A phylogenetic tree using full-length protein sequences was constructed to decipher the evolutionary relationships of NRT2 family genes among monocot (rice, barley, wheat, and maize) and eudicot (*Arabidopsis*, soybean, and papaya). The phylogenetic tree demonstrated that NRT2 family genes were divided into four clades (I to IV), including one outgroup gene named *ZmNRT2.3* with > 38.4% bootstrap values (Figure 1). Clade II has the fewest *NRT2* gene members (2), while clade IV consists of the most members (7), followed by clade III (6) and clade II (4). The NRT2 family members indicated a high degree of similarities. They were clustered into their respective monophyletic groups, reflecting the highly conserved nature of *NRT2* genes within the plant, particularly for monocot species in clades I and II and eudicot species in clade III. Based on phylogenetic analysis, eight sister pairs were discovered among them: (i) paralogous pairs of *OsNRT2.1/OsNRT2.2*, *ZmNRT2.1/ZmNRT2.2*, *AtNRT2.3/AtNRT2.6*, and *AtNRT2.1/AtNRT2.2*; and (ii) orthologous pairs of *TaNRT2.1/HvNRT2.6*, *AtNRT2.7/OsNRT2.4*, *GmNRT2/AtNRT2.5*, and *OsNRT2.3/HvNRT2.5*. Most paralogous pairs had high bootstrap support with > 99.9% bootstrap values, while bootstrap values of orthologous pairs were between 45.1% and 100%.

Functional Motifs of NRT2 Family Genes

Aside from conserved 60 amino acid residues, the rest of the protein sequence contains other motifs potentially involved in unknown functions or structural roles. Hence, twenty distinctive motifs were determined by analysing conserved amino acids ranging from 6 to 50 amino acid

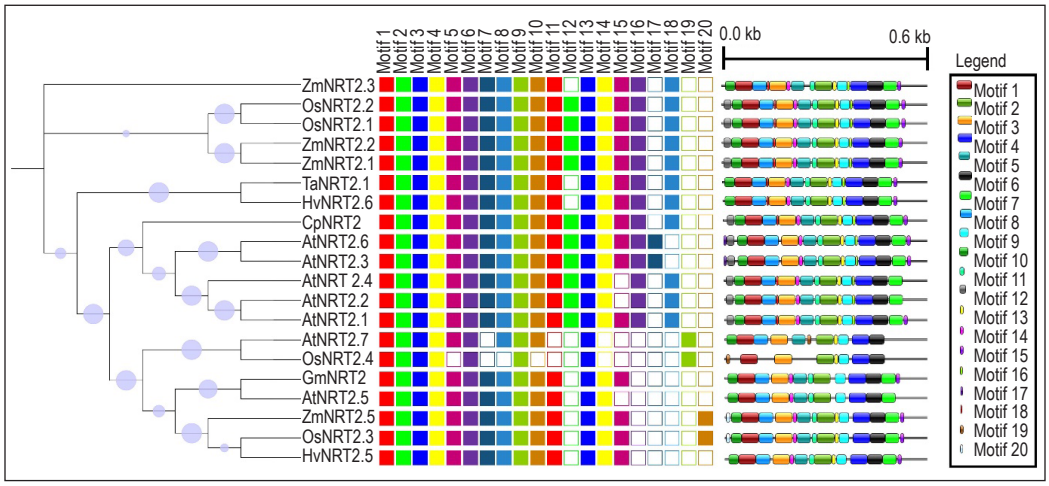

Figure 1. Illustrative diagram of motifs organisation for NRT2 family protein in the phylogenetic tree. The coloured box represents the presence and absence of distinctive motifs in NRT2 proteins. The arrangement of 6–50 amino acid motifs is shown in an orderly sequence of NRT2 proteins. The information on the motif is shown in Table 2

residues within the NRT2 protein sequences. The information is demonstrated in Figure 1 and Table 2. Generally, two or more motifs that have remained close to each other are considered domain regions. The function annotation of putative motifs was obtained from Pfam, PROSITE, and CDD searches. The conserved motif 1 to 11 comprises the nitrate transmembrane transporter domain, which is highly present in most NRT2 family proteins (Table 2). The orthologous pair of *AtNRT2.7/OsNRT2.4* had the least conserved motifs, with several motifs (motifs 7, 11, 12, 14 to 18, and 20)

Table 2
Twenty motifs of NRT family genes were identified using MEME Suite 5.3.3. Each motif consensus was annotated against CDD, Pfam, and PROSITE

Motif	Motif consensus	Motif length (aa)	Motif name
1	PHMRTFHLSWISFFTCFVSTFAAAPLVPIIRDNLNLTKADI GNAGVASVS	50	[1, 2, 4, 5]
2	YRTWIFVLLYGYSMGVELTTDNVIAEYFYDRFDLDLRTA GIIAASFGMAN	50	[1, 6]
3	RFLIGFSLATFVSCQYWMSTMFNSKIIGLVNGLAAGWG NMGGGATQLJMP	50	[1]
4	MVLFSFFAQAACGATFGVVPFVSRRSLGIISGMTGAGGN VGAGLTQLLFF	50	[1, 3, 6, 8]
5	TAWRIAFFVPGLLHVVMGILVLTLGQDLPDGNLRSLQK	38	[1, 6, 7]
6	TSSRYSTETGJEYMGIMIMACTLPVTLVHFPQWGSMFLP PSADA	44	[1]
7	TEEHYYASEWSEEEKSKGLHEASLKFAENSRSERGRRNVI	40	[1, 5, 6, 8, 9]
8	GSIFSRLAMGAVCDLLGPRYGCAFLIMLSAPTVFCMSFIDS	41	[1, 5, 6]
9	ARRFGMRGRLWNJWILQTAGGAFCIWLGR	29	[1]
10	EAADAKSKFDLPVDSEHKAKVFRLFSFAN	29	[1, 5]
11	AKDSFSKVLWYAVTN	15	[1]
12	EPGSSLHGVTGREQAFAFSVE	21	[5, 8]
13	VARPGGGLLSD	11	[6]
14	VYEAIRKCGAT	11	[10]
15	ATPPNNTPEHV	11	[10]
16	ASTLPTSV	8	[10]
17	MAHNHSNE	8	[4]
18	AGYIAV	6	[10]
19	EEEEKLVEEED	11	[10]
20	EFKPVAMZVE	10	[10]

Note.
[1]Nitrate transmembrane transporter; Provisional (cl30556)
[2]Leucine rich repeat (PF13516)
[3]Ubiquitinol-cytochrome C reductase Fe-S subunit TAT signal (PF10399)
[4]N-glycosylation site (PS00001)
[5]Casein kinase II phosphorylation site (PS00006)
[6]N-myristoylation site (PS00008)
[7]Major facilitator superfamily (MFS) (PS50850)
[8]Protein kinase C phosphorylation site (PS00005)
[9]Amidation site (PS00009)
[10]Unknown

found to be absent in both genes. Motifs 1 and 4 were putatively annotated as leucine-rich repeat, ubiquinol-cytochrome C reductase, Fe-S subunit, and twin-arginine translocation (TAT) signal, respectively. Meanwhile, motif five was identified as a short sequence region for a major facilitator superfamily that transports substrates across cell membranes. Several sites were annotated among the motifs, including the *N*-glycosylation site (motifs 1 and 17), casein kinase II phosphorylation site (motifs 1, 7, 8, 10, and 12), *N*-myristoylation site (motifs 2, 4, 5, 7, 8, and 13), protein kinase C phosphorylation site (motifs 4, 7, and 12) and amidation site (motif 7), which could play an essential role of protein structure. With motifs 1 to 11, it became clear that the most closely related clade members had similar motif distribution, suggesting functional similarities among NRT2 proteins within constructed phylogenetic trees.

Promoter Analysis of Putative Stress Responsive *OsNRT2.3*

The presence of *cis*-regulatory elements (CREs) in the promoter is critical for controlling gene transcription under particular conditions. As the functional motifs of *OsNRT2.3* are highly conserved among the orthologous and paralogous genes, responsive elements of *OsNRT2.3* was found to be implicated in vital biological processes. Therefore, the 2.0 kb promoter region of *OsNRT2.3* was analysed using PlantCare, resulting in four functional elements: hormone-, tissue-, stress-, and light-responsive elements. The promoter analysis revealed that stress-responsive elements were predominantly present in *OsNRT2.3*, with a total number of 19, followed by light-responsive elements (13), hormone-responsive elements (11), and tissue-specific elements (3). The information on these CREs and their locations are reported in Table 3 and Figure 2, respectively.

The identified CREs were discovered to be involved in numerous stress responses, for instance, Myb-binding site (MBS) (CAACTG), v-Myb myeloblastosis viral oncogene homolog (MYB) (CAACCA), and myelocytomatosis oncogenes (MYC) (GTTTAC) induced by drought; AU rich element (ARE) (AAACCA) responsive to anaerobic induction; GC-motif (GCCCCC) involved in oxygen deficiency; long terminal repeats (LTR) (CCGAAA) induced by low temperature; and wnt-responsive element 3 (WRE3) (CCACCTAC) responsive to wound. Interestingly, several hormone-responsive elements that are involved in various stresses were also discovered, including ABA-responsive element (ABRE) (CGGTGCG), ABA-responsive element 3a (ABRE3a) (TACGTG), and ABA-responsive element 4 (ABRE4) (ATGCAC) induced by abscisic acid and drought stress; CGTCA-motif (ACTGC) and TGACG-motif (TGACG) involved in methyl jasmonate responsiveness and biotic stress; ethylene-responsive element (ERE) (ATTTTAAA) responsive to ethylene; and gibberellic acid responsive element (GARE) (TCTGTTG) responsive to gibberellin. In addition, most of the light-responsible elements like Box-4

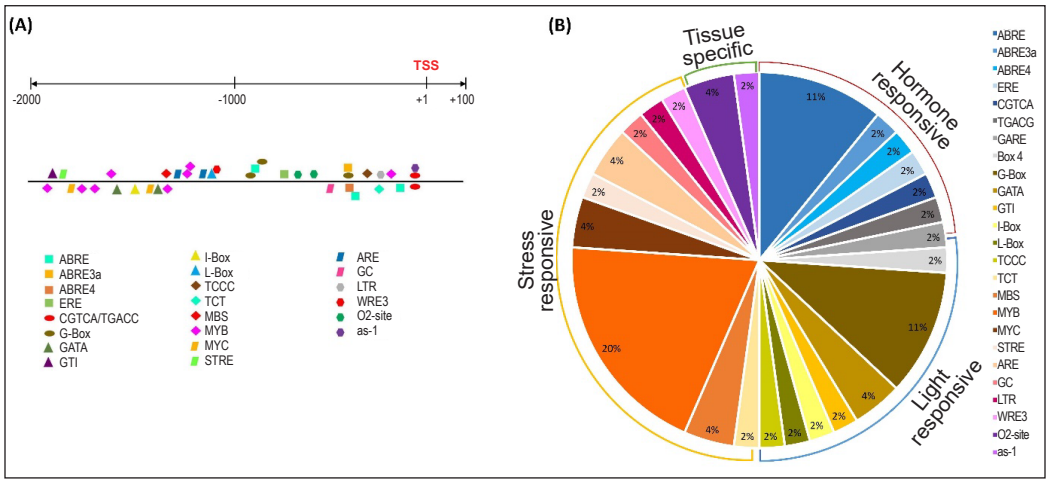

Figure 2. The information of plant stress-, hormone-, light- and tissue-specific-responsive elements (CREs) in the promoter regions of *OsNRT2.3*. (A) Predicted CREs in the promoter regions of *OsNRT2.3* gene; (B) Pie distribution of identified motifs in *O. sativa NRT2.3* gene from PlantCARE, based on their biological functions

Table 3
Description of putative cis*-acting regulatory elements in* OsNRT2.3 *promoter region from the PlantCARE database*

Type	Element	Sequence (5'-3')	Function	Abundance of element
Hormone-responsive element	ABRE	CGGTGCG	*Cis*-acting regulatory element involved in the ABA responsiveness	5
	ABRE3a	TACGTG	*Cis*-acting regulatory element involved in the ABA responsiveness	1
	ABRE4	ATGCAC	*Cis*-acting regulatory element involved in the ABA responsiveness	1
	ERE	ATTTTAAA	ethylene-responsive element	1
	CGTCA-motif	ACTGC	*Cis*-acting regulatory element involved in the MeJA-responsiveness	1
	TGACG-motif	TGACG	*Cis*-acting regulatory element involved in the MeJA-responsiveness	1
	GARE	TCTGTTG	Gibberellin responsive element	1
Light-responsive element	Box-4	ATTAAT	Part of a conserved DNA module involved in light responsiveness	1
	G-box	TAAACGTG	*Cis*-acting regulatory element involved in light responsiveness	5
	GATA-motif	GGAAGAGGAA	Part of a light-responsive element	2
	GTI-motif	GGTTAA	Light responsive element	1
	I-box	TCGGAGTAGAA	Part of a light-responsive element	1
	L-box	ATCCCACCT	Part of a light-responsive element	1
	TCCC-motif	TCTCCCT	Part of a light-responsive element	1
	TCT-motif	CATTCT	Part of a light-responsive element	1

Table 3 *(continue)*

Type	Element	Sequence (5'-3')	Function	Abundance of element
Stress-responsive element	MBS	CAACTG	MYB binding site involved in drought-inducibility	2
	MYB	CAACCA	MYB recognition site involved in drought responsiveness	9
	MYC	GTTTAC	MYB recognition site involved in drought responsiveness	2
	STRE	AGGGG	Stress-responsive element	1
	ARE	AAACCA	*Cis*-acting regulatory element essential for the anaerobic induction	2
	GC-motif	GCCCCC	Enhancer-like element involved in anoxic specific inducibility	1
	LTR	CCGAAA	*Cis*-acting element involved in low-temperature responsiveness	1
	WRE3	CCACCTAC	Wound responsive element	1
Tissue-specific element	O2-site	GATGATGTGG	*Cis*-acting regulatory element involved in zein metabolism regulation	2
	As-1	TGACG	*Cis*-acting regulatory element involved in the root-specific expression	1

(ATTAAT), G-box (TAAACGTG), GATA-motif (GGAAGAGGAA), GT1-motif (GGTTAA), I-box (TCGGAGTAGAA), L-box (ATCCCACCT), TCCC-motif (TCTCCCT), and TCT-motif (CATTCT) are responsible for responding to a broad spectrum and specific wavelength of light. In the case of tissue-specific elements, opaque 2 (O_2-site) (GATGATGTGG) and as-1 (TGACG) are essential in zein metabolism regulation and root-specific expression, respectively.

Identification of *OsNRT2.3* Co-Expressed Genes and GO Annotation Analysis

Co-expression analysis is widely used to perceive transcription regulators in rice. It was used as a query gene to retrieve the information of the co-expressed genes from Rice Expression Database (RED), with the selected Pearson's *r* value greater than 0.85 to exploit the transcription regulation of the *OsNRT2.3* gene. As a result, 18 genes were identified to be significantly co-expressed with *OsNRT2.3* (Table 4). Nine co-expressed genes were assigned to numerous GO annotations focusing on lipid transport and localisation, cellular macromolecule metabolic processes, stress response, and transcription regulation. Most of the genes encode proteins in the nucleus and membrane. The molecular functions discovered are transcriptional regulation, binding, protein kinase activity, transporter, protein dimerisation, oxidoreductase, and hydrolysis. These findings imply that annotated co-expressed genes may be associated with transport and localisation.

Table 4
Co-expressed genes of OsNRT2.3 *(*LOC_Os01g50820/Os01g0704100*) obtained from Rice Expression Database (http://expression.ic4r.org)*

MSU ID	Gene ID	Gene name	Protein name	Pearson's *r* value
LOC_Os01g22920	Os01g0332200	*GA2ox2*	Gibberellin 2-beta-dioxygenase 2	0.921323
LOC_Os01g42370	Os01g0609200	*PDR11*	Pleiotropic drug resistance 11	0.977791
LOC_Os02g26950	N/A	*Os02g0468900*	Hypothetical protein	0.873701
LOC_Os02g40710	Os02g0620500	*AMT1.3*	Ammonium transporter 1.3	0.899457
LOC_Os03g19375	Os03g0306700	*bZIP27*	bZIP transcription factor 27	0.918212
LOC_Os03g22390	N/A	*Os03g0344166*	Universal stress protein family protein	0.854134
LOC_Os03g46470	Os03g0667500	*IRT1*	Iron-regulated transporter 1	0.92206
LOC_Os03g58670	N/A	*Os03g0801200*	Plant lipid transfer/seed storage/ trypsin-alpha amylase inhibitor domain-containing protein	0.982927
LOC_Os05g28770	Os05g0355700 Os05g0355800	*GCRP9*	Glycine and cysteine-rich family protein	0.936195
LOC_Os05g29000	Os05g0358101	*Os05g0358101*	Expressed protein	0.861334
LOC_Os06g24460	N/A	*PDR2*	Pleiotropic drug resistance protein 2, putative	0.871721
LOC_Os06g30860	Os06g0504900	*WRKY31*	WRKY transcription factor 31	0.934795
LOC_Os07g24930	N/A	*N/A*	Retrotransposon protein, putative, Ty3-gypsy subclass	0.960406
LOC_Os07g24940	Os07g0431160	*Os07g0431160*	Transposon protein, putative, CACTA, En/Spm sub-class	0.870374
LOC_Os07g25300	N/A	*N/A*	Retrotransposon, putative, centromere-specific	0.972477
LOC_Os08g31890	Os08g0413200	*Os08g0413200*	Hypothetical protein	0.927934
LOC_Os08g44800	N/A	*N/A*	Hypothetical protein	0.874997
LOC_Os12g03830	Os12g0132500	*ZIFL9*	Zinc-induced facilitator-like 9	0.983711

From the co-expression network in Figure 3A, *OsNRT2.3* is co-expressed with several important genes in NUE; particularly genes that encode proteins involved in transport, such as zinc-induced facilitator-like 9 (*OsZIFL*) and ammonium transporter 1.3 (*OsAMT1.3*), plant lipid transfer/seed storage/trypsin-alpha amylase inhibitor domain-containing protein (*Os03g0801200*), in lipid transport and localisation, and universal stress protein (*Os03g0344166*) in stress response. *OsNRT2.3* is also co-expressed with fungus-inducible *OsbZIP27* and pathogen-inducible *OsWRKY31*, exhibiting these defense-related transcription factors (TFs) have a causal role in putatively controlling the transcriptional regulation of *OsNRT2.3* and its co-expressed genes under stresses. Based on bibliomic searching, the unannotated co-expressed genes, *gibberellin 2-beta-dioxygenase 2* (*OsGA2ox2*), enhanced

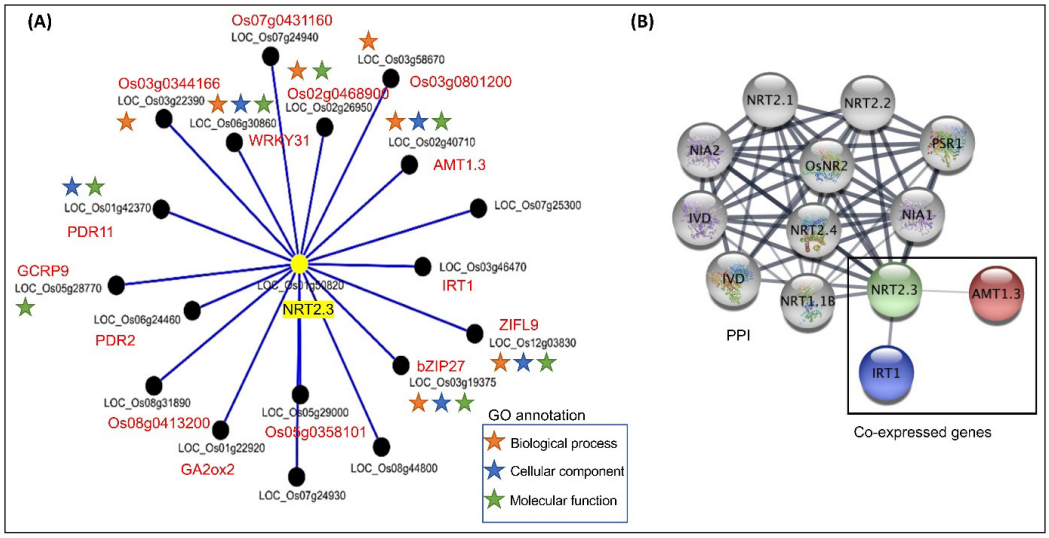

Figure 3. Interaction of (A) co-expressed genes with *OsNRT2.3* obtained from Rice Expression Database (http://expression.ic4r.org) and (B) gene network inference of *OsNRT2.3* and its co-expressed genes using PPI data from STRING database (https://string-db.org)

lodging resistance in rice. In contrast, *iron-regulated transporter 1* (*OsIRT1*) was critical in cadmium uptake and translocation during iron deficiency. Although knowledge of the reported co-expressed genes is still limited, these genes may play a possible role in directly or indirectly being involved in NUE in rice.

In silico Inference of Functional *OsNRT2.3* Co-Expressed Genes by PPI

To strongly support the co-expression analysis at the functional level, the PPI of *OsNRT2.3* and 18 co-expressed genes was conducted using StringApp, a Cytoscape plugin. Therefore, the *OsNRT2.3* PPI was discovered to consist of 13 genes with 50 interactions. This data was used to infer the function of *OsNRT2.3* co-expressed genes based on the interaction gene lists of a priori knowledge. From the STRING data, the *OsNRT2.3* interacts with *OsAMT1.3* and *OsIRT1*, which regulated the response of NH_4^+ and Fe uptake, exhibiting the interplay of the transporter proteins in rice under nutrient-deficient soil (Figure 3B). Despite the absence of the other co-expressed genes in PPI data, their association with several biological processes, including transport, response to stress, protein phosphorylation, and cellular macromolecule metabolic process, may suggest them as new candidate genes occurred in coordinating regulation of macromolecule metabolism and other critical physiological functions.

Nine proteins were also found functionally related to *OsNRT2.3* based on their gene ontology annotation information, whereas the other nine proteins' functions were classified as unknown (Figure 3A). The interacting annotated partners of *OsNRT2.3* comprised four nitrate transporters (*OsNRT2.1*, *OsNRT2.2*, *OsNRT2.3*, and *OsNRT1.1B*), three nitrate reductase (*OsNR2*,

OsNIA1, and *OsNIA2*), promoter of shoot regeneration (*OsPSR1*), and isovaleryl-CoA dehydrogenase (*OsIVD*). These proteins are associated with several NUE-related processes, including N utilisation, nitrate assimilation, and response to nitrate, ammonium, and chlorate, having similar biological processes to the *OsNRT2.3* protein.

Expression Patterns Analysis of *OsNRT2.3* Co-expressed Genes

To discover the transcript level of the *OsNRT2.3* gene and its co-expressed genes in the specific tissue, the similar expression profiles of selected genes were visualised in various tissues of rice using the ePlant, a data visualisation tool in the Bio-Analytic Resource for Plant Biology (BAR) database. Interestingly, the expression level of co-expressed genes, *OsAMT1.3*, *OsbZIP27*, *OsIRT1*, and *OsZIFL9*, were discovered to be relatively similar to *OsNRT2.3* in all 15 tissues. The transcript of all five genes demonstrated a higher expression level in the seedling root, with an average expression level from 850.99 to 12,511.73 (Figure 4). *OsIRT1* had the highest expression level in

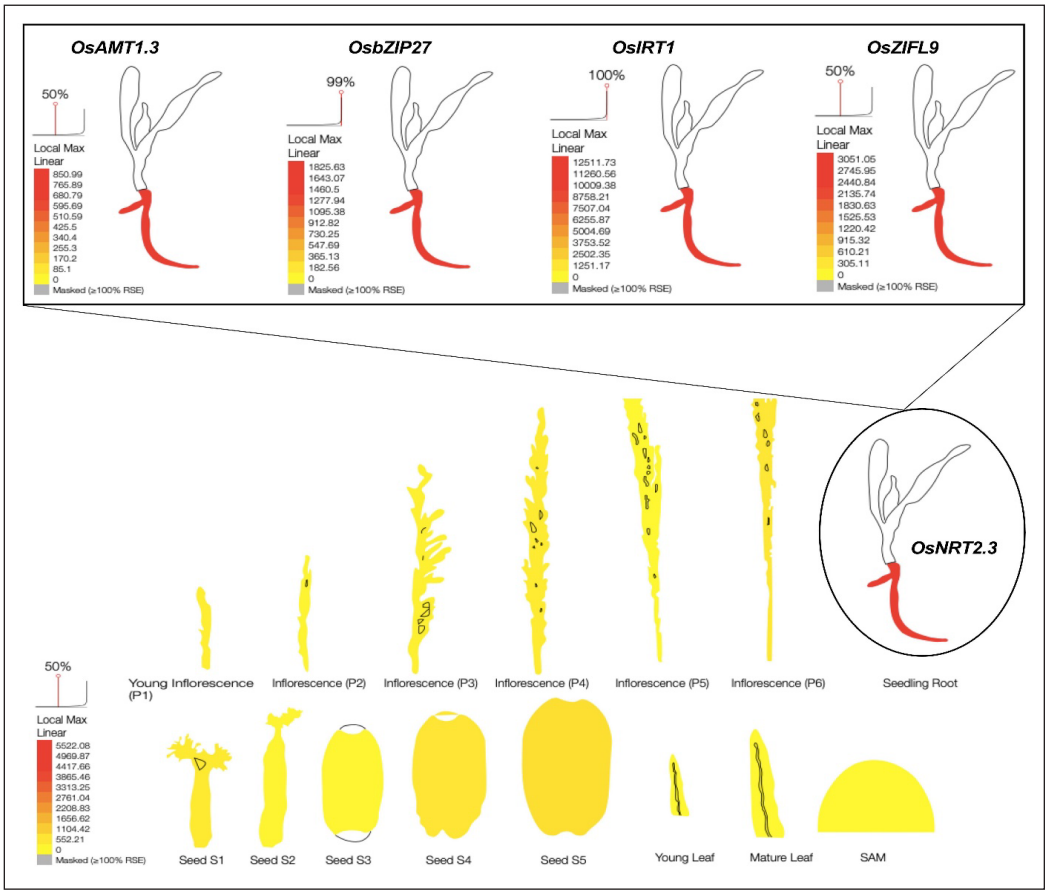

Figure 4. Expression levels of four co-expressed genes: *OsAMT1.3*, *OsbZIP27*, *OsIRT1*, and *OsZIFL9* with *OsNRT2.3* in 15 tissue-specific obtained from ePlant (http://bar.utoronto.ca/eplant/)

the seedling root, followed by *OsNRT2.3* (5522.08), *OsZIFL9* (3,051.05), *OsbZIP27* (1,825.63), and the lowest is *OsAMT1.3*. The particular genes may reflect their crucial role in the absorption and transportation of micronutrients available via root in rice.

The expression patterns of genes were observed to reveal more information on the potential involvement of co-expressed genes in N response using a rice whole-genome Affymetrix GeneChip array. Under different treatments of N response in leaf and root, *OsNRT2.3*, *OsAMT1.3*, and *OsIRT1* had similar expression patterns in the root and were highly expressed under low N and root-induced N (Figure 5). The decreased gene expression level under normal N conditions and root-reduced N may also suggest their importance in balancing the N uptake and utilisation due to nitrification in aerobic soils. Therefore, the involvement of the *OsAMT1.3* and *OsIRT1* were inferred to play a significant role in NUE and provided two putative candidates, *OsbZIP27* and *OsZIFL9*, to interact with *OsNRT2.3* during the control of N transportation in rice.

DISCUSSION

The nitrate transporter (NRT) is a vital gene family required for nitrate (NO_3^-) absorption and transport for plant growth. In addition, NRT is essential in motioning water and solutes across the cell membrane. A recent study about the importance of the NRT family was reported, such as the *NRT2* gene responsive to stresses in rapeseed (*Brassica napus* L.) (J. Tong et al., 2020) and the genetic effects of *NRT* genes in Chinese white poplar (*Populus tomentosa*) (Zhao et al., 2021). However, the roles of *OsNRT2.3* and its co-expressed genes in the association of drought and NUE in rice remain elusive, particularly in reporting the potential drought-related genes

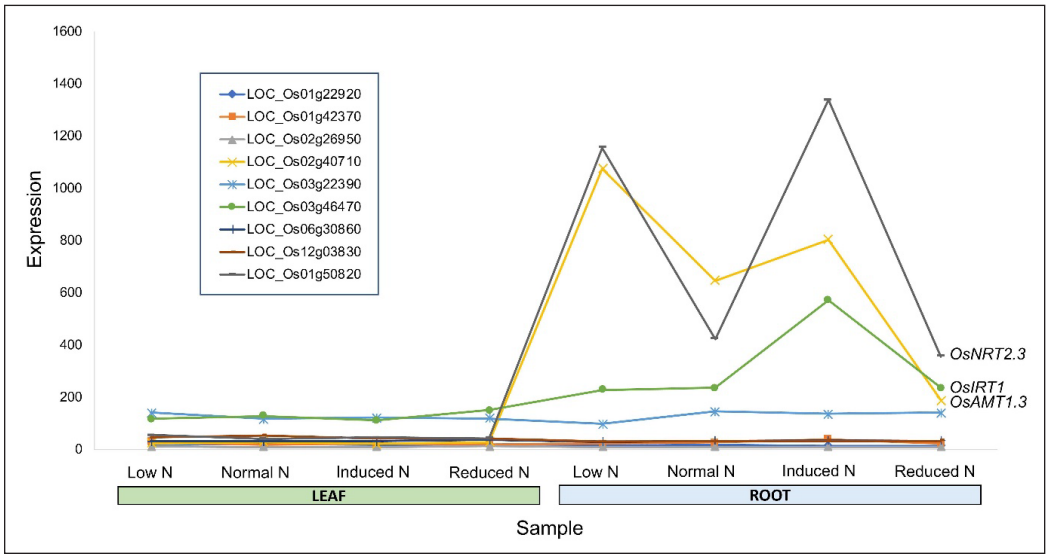

Figure 5. Expression patterns of *OsNRT2.3*, *OsAMT1.3*, and *OsIRT1* in the root and leaf under the different N conditions

during NUE. Thus, our study presents the *in silico* analysis of four *OsNRT2*, seven *AtNRT2*, four *ZmNRT2*, two *HvNRT2*, one *GmNRT2*, one *CpNRT2*, and one *TaNRT2* to comprehend the evolutionary relationship of *OsNRT2.3* with other *NRT2* genes of monocot and eudicot. Twenty *NRT2* genes are divided into four monophyletic groups in the phylogeny by introducing eight sister pairs of ortholog and paralog members. By analysing the distribution of the motif of *NRT2* genes, most of these genes were highly conserved by considering the motifs remaining close to each other in nitrate transmembrane transporter domain regions.

Nevertheless, some *NRT2* genes have distinct motif distributions, exhibiting that the protein domain likely evolved at different substitution rates to adapt to the environment (Schaeffer et al., 2016). The different lengths of the NRT2 protein sequences for the sister pairs may also suggest the possible insertion and/or deletion events throughout evolution that could further understand the functional diversity of genes encoding NRT2 proteins (Abdullah-Zawawi et al., 2021). Therefore, we found that *OsNRT2.3* is in a similar sub-clade phylogeny to *ZmNRT2.5* and *HvNRT2.5* and consists of similar motifs, which indicates that these genes may serve similar functions. From a previous study, H. Wang et al. (2017) discovered that *ZmNRT2.5* enhances the rapid accumulation of amino acids and increases N uptake in roots during a drought. The survival rate of transgenic lines of *OsNRT2.3* is not significantly different from *OsNRT2.1* transgenic lines under drought conditions and is considered relatively regulated in increasing drought tolerance in rice (Chen et al., 2019).

The CREs play a significant role in gene regulation by controlling a substantial gene network in biological function. The presence of prominent CREs in the promoter region of the *OsNRT2.3* gene highlights the potential involvement of the responsive gene in stress and hormone based on the predominant number of CREs that appeared in *OsNRT2.3,* such as MYB and ABRE. A study by M. Wang et al. (2020) reported that *NRT2.3* is induced by ABA signals in wheat roots through the transcript abundance of *TaNRT2.3* after being treated with nitrate. An abundant MYB element in the promoter region of drought-responsive genes also revealed its higher root expression in rice and other crops (Khan et al., 2017). Therefore, the co-expression interaction of the *OsNRT2.3* gene is observed to fully comprehend the regulation of the respective gene that controls other genes in NUE.

A combinatorial analysis of gene co-expression and gene expression pattern revealed that four co-expressed genes, including *OsAMT1.3*, *OsbZIP27*, *OsIRT1*, and *OsZIFL9*, are strongly co-regulated with *OsNRT2.3* in response to N availability based on Pearson correlation coefficient, PCC > 0.85 and expression pattern similarity. Furthermore, the expression levels of these five genes were found to be highly expressed in seedling roots. *OsAMT1.3* and *OsIRT1* showed similar expression patterns with *OsNRT2.3* under low and normal N conditions and root-induced and

root-reduced N conditions. *OsbZIP27* and *OsZIFL9*, on the other hand, only expressed similarly with *OsNRT2.3* under low and normal N conditions, suggesting that all these four genes may play an important role in the correlation between drought and NUE.

Plants develop various adaptation strategies, including tolerance mechanisms and N-use optimisation due to the drought response. Under drought stress, plant roots will absorb more water from the soil to seize an optimal amount of N for metabolism (Waraich et al., 2011), affecting the N assimilation process and resulting in the declination of NUE (Hoang et al., 2019). *OsAMT1.3* is a root-specific gene regulated by nitrogen supply and is also involved in enhancing rice growth and carbon-nitrogen metabolic status (Bao et al., 2015; Sonoda et al., 2003). There is no clear evidence of *OsIRT1* being involved in NUE; however, it was reported that *OsIRT1* is a preferential Fe (II) transporter whose function in NUE can be characterised using the reverse genetics approach (Z. Zhang et al., 2020). OsZIFL9, a Zn transporter, is known to have a crucial role in improving nutrient status in rice, although its status in N management is still understudied (Awasthi et al., 2021). Therefore, deciphering the correlations of potential drought-related genes' function with NUE is challenging but important. It is suggested that optimising N use could support drought tolerance in rice and require further validation of the genes in future studies.

Generally, in the flooded soil, N is usually distributed as NH_4^+, while in the upland, it is converted into NO_3^- due to nitrification by aerobic soils (Kabange et al., 2021; Qian et al., 2004). An NH_4^+ transporter, OsAMT1.3, acts as a signal sensor for regulating NUE under low NH_4^+ conditions (Ferreira et al., 2015). A recent study also suggested that *OsAMT1.3* co-expressed with the *NRT* gene after applying potassium chlorate ($KClO_3$) to simulate a drought (Kabange et al., 2021). *OsZIFL9* and *OsIRT1*, on the other hand, are reported to play a potential role in alleviating Fe deficiency by NH_4^+ in calcareous soils. In the case of *OsIRT1*, this transporter gene is induced by low Fe conditions, and ethylene (ET) will enhance the expression of the respective gene in the roots uptake system of Fe^{3+} and Fe^{2+} phytosiderophore (Ishimaru et al., 2006; J. Wu et al., 2011). Recently, García et al. (2021) discovered that ethylene (ET) plays a significant role in regulating crosstalk between the nutrient deficiency through activation of the ET transduction pathway for facilitating the plant response, particularly by enhancing the transporter activity. The assimilation of NH_4^+ and NO_3^- are different in apoplastic pH, influencing the uptake and utilisation of Fe in rice. First, NO_3^- reduces the xylem alkalinity by delivering Fe into the cell wall. Secondly, NH_4^+ enables the reduction of aerenchyma division, which ultimately adjusts the water uptake capacity of Fe from roots to different parts of the rice (X. Zhang et al., 2019). The above findings proposed that the presence of

OsNRT2.3, coupled with the *OsIRT1* activity in roots, would designate the prevalence of Fe uptake during NO_3^- and NH_4^+ transport.

A previous study showed that *OsbZIP27*, a bZIP-type transcription factor, was induced by dehydration stress under a mild water deficit of rice (Hossain et al., 2010). The bZIP proteins have a DNA-binding specificity for ACGT-containing DNA sequence motifs (Izawa et al., 1993). Despite the lack of a specific study of the *OsbZIP27* function in regulating *OsNRT2.3*, ACGT-binding specificity of G-box and ABRE3a elements were discovered in the *OsNRT2.3* promoter region. It reflects that OsbZIP27 proteins may activate transcription of ABA-induced *OsNRT2.3* by binding to the ABREa element for drought tolerance of rice. In addition, previous studies have also demonstrated that roots overexpressing *ZIFL* genes have significantly increased by Zn and Fe (Sharma et al., 2019). Under Fe-deficient conditions, FER-LIKE FE DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (OsFIT) and its interacting partner, iron-related bHLH transcription factor 2 (*OsIRO2*), play a critical role in regulating Fe-acquisition genes for Fe homeostasis in the roots and leaves, including *OsZIFL9* that increased in expression level (Liang et al., 2020).

Interactions between essential nutrients, such as iron (Fe), nitrogen (N), sulfur (S), and phosphorus (P), were reported to influence transport, homeostasis, and assimilation processes for proper growth and development in plants (Kumar et al., 2021). For example, according to Singh et al. (2018), the concentration of Fe, Zn, and protein in wheat grains increased due to the interaction of Fe and Zn with N. Likewise, the concentration of micronutrients like Cu, Zn, Mn, and Na increased in the belowground tissue of *Bothriochloa ischaemum* when exposed to a high amount of N (Ai et al., 2017). Thus, our study demonstrates that the NUE system's potential drought-related genes, *OsAMT1.3*, *OsbZIP27*, *OsIRT1*, and *OsZIFL9,* are highly associated with *OsNRT2.3*, and *OsZIFL9* and *OsIRT1* might be key players in the interaction of macronutrient N and micronutrient Fe in rice.

CONCLUSION

Transporters involved in the uptake, transport, and re-translocation of nitrogen (N) are important targets in breeding programmes to combat environmental stress, particularly drought. Computational dissection of potential drought-related genes from *NRT2* will greatly advance our knowledge of the relationship between NUE and drought. This study has investigated 20 selected *NRT2* genes of various plants, and their motif compositions are mostly similar and conserved within the nitrate transmembrane transporter domain across the monophyletic group. In rice, *NRT2.3* genes interacted specifically with stress and phytohormone responsiveness elements. The correlation between genes has emerged as a growing research focus in crop improvement. The interaction of four potential genes, *OsAMT1.3*, *OsbZIP27*, *OsIRT1*, and *OsZIFL9*, with *OsNRT2.3*, provides an alternative explanation of the

relationship between NUE and drought response in rice. This knowledge will assist the future investigation, such as genome editing via the CRISPR-Cas9 system and reverse genetics, to understand better the function of potential genes in the N uptake system and drought stress. The present findings not only identified the genes that potentially govern drought and NUE but can also expedite the discovery and molecular validation of the biologically important gene in breeding strategies.

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