

The multiple stress-tolerant gene *OsSRDP* exhibited nitrogen stress adaptation by transcriptional regulation of downstream genes, regulation of plant secondary metabolism and carbon-nitrogen metabolic reprogramming

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Research Article

Keywords: Nitrogen Use Efficiency, N-stress, PacBio sequencing, Transcriptome analysis, *OsSRDP*, DUF740 gene family, Rice

Posted Date: October 15th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11349431/v1>

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Additional Declarations: The authors declare no competing interests.

Abstract

Nitrogen is the key macronutrient indispensable for rice cultivation. Despite rice having a low Nitrogen Use Efficiency (NUE) of 30–40%, a huge amount of nitrogenous fertiliser application continues, leading to environmental pollution through leaching. Hence, improving the NUE of rice is crucial for proper nutrient usage and reducing environmental loss. Two rice genotypes Pusa Sugandh 2 (PS2) and a transgenic rice (PS2-SRDP) in the PS2 background with overexpressed *OsSRDP* were grown under two treatments of Nitrogen source, 8 mM (N-optimum) and 0.08 mM (N-stress) conditions for 28 days. Long-read sequencing of RNA isolated from the shoot and root tissues of control and treated rice genotypes was performed on the PacBio Sequel II system. We identified 306 and 221 differentially expressed genes (DEGs) across all comparisons in the root and shoot, respectively. We observed 7 and 25 DEGs specific to N-stress conditions in PS2-SRDP. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of the selected DEGs differentially expressed between PS2-SRDP and PS under low N stress revealed carbon metabolism, secondary metabolite, amino acid metabolism, glycolysis, pyruvate metabolism, alpha-linolenic acid pathways and fatty acid degradation as the major enriched pathways. Cellular components were enriched among the Gene Ontology terms. Protein-protein Interaction network study also supports to the identified pathways. This study explores the effects of *OsSRDP* under N-stress through transcriptome analysis of transgenic rice. Differential expression analysis revealed the downstream genes those expressions modified under the effect of *OsSRDP* under low N-stress. These genes can be further explored for their interactions with *OsSRDP* and their roles in low-nitrogen metabolism, with the aim of improving NUE in crops.

1. Introduction

Nitrogen (N) is an essential macronutrient used in commercial rice production, since modern-agriculture deploys nitrogenous fertilisers responsive cultivars (Galloway et al., 2008). In the cultivation of commercial rice crop varieties, on average 100–150 kg N is applied as fertiliser per hectare of area, more than half of which is lost to the environment (soil, water and atmosphere) due to poor nitrogen use efficiency (NUE) (Hakeem et al., 2011). These losses account for higher costs of cultivation and cause environmental pollution. Hence, optimising N management is essential for minimising N losses from soil (Ren et al., 2022).

Other than management practises, exploitation of plant genetic potential is important to enhance NUE, but plant NUE is controlled by several component traits like uptake, assimilation, allocation and remobilisation (Xu et al., 2012), and a detailed understanding is possible only when the underlying genes in these processes and their interactions are known.

One such gene, *Os04g0690500* from rice, was primarily reported to be differentially expressed between the Nagina 22 and IR64 rice genotypes under different abiotic stress treatments. The gene showed upregulation under drought stress in both genotypes (Jayaraman et al., 2021) and contained the DUF740 domain. Subsequently, the gene was isolated from the drought- and heat-tolerant genotype Nagina 22

and functionally characterised by expressing it under the stress-inducible promoter, AtRd29, in the drought-susceptible rice genotype Pusa Sugandh 2 (PS2). The transgenic rice conferred multiple abiotic stress tolerances and also showed tolerance to rice blast. The gene was named OsSRDP (*Oryza sativa* Stress Responsive Drought Protein) after characterisation. Co-expression analysis of this gene using RiceFRIEND revealed that, beyond its role in abiotic stress tolerance, there was an interaction of this gene with those other genes involved in N metabolism in rice (Jayaraman et al., 2023), prompting us to study the role of this gene under low N stress.

Low N-stress was applied in this study to determine the N-stress response of OsSRDP by comparing wild-type PS2 and transgenic PS2 (PS2-SRDP). However, to identify the interacting partners, complete RNA sequencing and transcriptome analysis are important, as the complexity of the crop transcriptome regulates growth, development and responses to various abiotic and biotic cues in the environment. Also, structural and functional genomics of plants are represented by their high-quality genome and transcriptome data (Hasan et al., 2022).

While doing RNA sequencing, Oxford Nanopore sequencing and Pacific Biosciences SMRT long-read sequencing revolutionised transcriptome analysis by providing various advantages over short-read sequencing technologies (Zhao et al., 2019). For instance, short-read sequencing technologies require the RNA or cDNA to be fragmented, which is not needed in the case of PacBio and Oxford Nanopore sequencing, where the entire cDNA molecules are sequenced end-to-end (Cui et al., 2020). For these advantages, long-read RNA sequencing technology has been exploited for the transcriptomic profiling of various plants (Chen et al., 2023; Schaarschmidt et al., 2020; Zhang et al., 2022). The full-length transcript sequence obtained is beneficial for gene function, gene expression and regulation studies over short-read Illumina sequences (Chen et al., 2019). Hence, in this study, we have carried out RNA sequencing of PS2 and PS2-overexpressing lines for OsSRDP (PS2-SRDP), treated under N-stress conditions, using the PacBio Sequel II sequencing platform followed by transcriptome analysis. We finally wanted to establish the effect of *OsSRDP* in the rice under chronic N stress treatment, and also the response of other associated genes which altered different metabolic pathways.

2. Materials and methods

1.1. Experimental material

PS2 is a semi-dwarf, high-yielding aromatic rice variety suitable for cultivation in irrigated areas. PS2 rice variety and transgenic seeds of PS2-SRDP (Jayaraman et al., 2023) were grown in vermiculite and perlite mixture (2:1) provided with Yoshida media containing 8.0 mM (Optimum) and 0.08 mM (stressed) N source following the protocol of (Sinha et al., 2018). Shoot and root tissues were sampled from control (N-optimum) and treated (N-stress) plants 28 days after sowing (DAS) from both PS2 and PS2-SRDP plants, snap-frozen using liquid nitrogen and stored at -80 °C for later use. In total 16 samples were progressed for RNA sequencing (2 genotypes × 2 treatments × 2 tissues × 2 biological replicates).

1.2. Morpho-physiological data

Shoot length (SL), shoot fresh weight (SFW), shoot dry weight (SDW), root length (RL), root fresh weight (RFW) and root dry weight (RDW) of both PS2 and PS2-SRDP seedlings were measured at 28 DAS under N-optimum as well as N-stress conditions (Sinha et al., 2018). Chlorophyll and carotenoid content of the leaf samples were estimated according to Hiscox & Israelstam (1979).

1.3. RNA isolation, library preparation and sequencing

Total RNA isolation was carried out using Trizol (RNAiso Plus) and purified by the MN NucleoSpin RNA cleanup kit as per the manufacturer's protocol. RNA quality and quantity check, RNA integrity evaluation, cDNA synthesis, subsequent cDNA amplification and purification was performed according to (Thirugnanasambandam et al., 2023)). Subsequently, cDNA was pooled at equimolar concentration based on the average fragment size read on Agilent Bioanalyzer 2100. DNA damage and end repair were carried out for SMRTbell library preparation, followed by overhang adapter ligation. SMRTbell Express Template Preparation Kit 2.0 (Pacific Biosciences, California, USA) used for the library construction as per the manufacturer's protocol. Pronex beads (Promega, Wisconsin, USA) was used for library purification and the library size was estimated using Bioanalyzer (Agilent Technologies, California, USA). Annealing binding complex (ABC) from the library was performed. Finally, after polymerase binding and primer annealing, primer V4 (85 pM OPLC) of the library was loaded onto a SMRT cell containing 8 M ZMW and sequenced on the PacBio Sequel II system in CCS/HiFi mode.

1.4. Data processing

The PacBio software package SMRT Link 10.2 was used for data analysis. Briefly, circular consensus sequence (CCS) reads were generated from the raw read subread dataset using ccs SMRT tool. The CCS reads were refined using IsoSeq3 refine to obtain the Full Length Non-Chimeric (FLNC) reads for each sample. FLNC reads were clustered at isoform level and classified into high- and low-quality reads. Mapping of the HQ reads was done with the pbmm2 minimap aligner using the reference genome *Oryza sativa* IRGSP1.0 from RAP-DB (Rice Annotation Project Database).

1.5. Differential gene expression, KEGG pathway and GO enrichment analysis

DESeq2 was used for the identification of differentially expressed genes (DEGs) for the following pairwise comparisons (Wang et al., 2010).

1. PS2-SRDP Control Root (SRDPCR) vs PS2 Control Root (PS2CR)
2. PS2-SRDP Control Shoot (SRDPCS) vs PS2 Control Shoot (PS2CS)
3. PS2 Nitrogen Stress Root (PS2NR) vs PS2 Control Root (PS2CR)
4. PS2 Nitrogen Stress Shoot (PS2NS) vs PS2 Control Shoot (PS2CS)
5. PS2-SRDP Nitrogen Stress Root (SRDPNR) vs PS2 Nitrogen Stress Root (PS2NR)
6. PS2-SRDP Nitrogen Stress Shoot (SRDPNS) vs PS2 Nitrogen Stress Shoot (PS2NS)
7. PS2-SRDP Nitrogen Stress Root (SRDPNR) vs SRDP Control Root (SRDPCR)
8. PS2-SRDP Nitrogen Stress Shoot (SRDPNS) vs SRDP Control Shoot (SRDPCS)

Differential gene expression was determined based on the criteria of $|\log_2(\text{Fold change})| \geq 1$, $p\text{-value} < 0.05$ and $\text{padj-value} < 0.05$. The total number of differentially expressed gene comparisons was represented as a Venn diagram using Venn2.1. Functional annotation of DEGs was carried out through the Kyoto Encyclopedia of Genes and Genomes (KEGG) gene database. Metabolic pathway enrichment analysis was done using ShinyGO v0.82 (<https://bioinformatics.sdstate.edu/go>). Gene Ontology (GO) for the DEGs was analysed using Singular Enrichment Analysis (SEA) in agriGO v2 (https://systemsbiology.cau.edu.cn/agriGOv2/classification_analysis.php?category=Plant&&family=Poaceae).

1.6. Protein-Protein Interaction (PPI) network analysis

PPI network constructed from the DEGs mapped to the KEGG pathway using STRING Version 12.0. (<https://string-db.org>) individually for the root and shoot data with a high confidence score of 0.7 and exported to Cytoscape v3.10.4 for further visualisation.

1.7. Validation of RNA-Seq data by RT-qPCR

Both PS2 and PS2-SRDP were grown under N-optimum and N-stress conditions, and total RNA was isolated from the shoot and root samples as mentioned earlier. First-strand cDNA synthesis, primer designing and qPCR were performed following Sevanthi et al., (2021). Rice actin *OsACT1* was taken as internal reference gene. All primer sequences used in this study are provided in Table S1.

1.8. Statistical analysis

All samples were taken in triplicates for morpho-physiological data from all the samples. Two-way Analysis of Variance (ANOVA), multiple comparison analysis by Tukey's test and graphs were generated using GraphPad Prism 11.

2. Results

3.1 Morpho-physiological data

Morpho-physiological parameters were recorded from N-optimum and N-stressed plants of both PS2 and PS2-SRDP at 28 DAS (Fig. 1). When we compared PS2 and PS2-SRDP, most of the morphological parameters were on par under both N-optimum and N-stress conditions. Reduced nitrogen supply significantly increased the RL in PS2 (14.7 cm to 19.3 cm) but not in the PS2-SRDP (Fig. 2A). RFW was reduced substantially in both the PS2 (0.51 g to 0.21 g) and PS2-SRDP (0.66 g to 0.2 g) rice compared to their control counterparts (Fig. 2B). Similarly, RDW also reduced in both PS2 (0.06 g to 0.03 g) and PS2-SRDP (0.08 g to 0.03 g) seedling (Fig. 2C). In case of SL, PS2 (49.2 cm to 30.3 cm) and PS2-SRDP (50.2 cm to 29.0 cm) showed similar reduction under N-stress condition (38–42%) (Fig. 2D). We found SFW and SDW also showed similar pattern in both the PS2 and PS2-SRDP (Fig. 2E and 2F). Chlorophyll and carotenoid content were significantly higher in PS2-SRDP than in PS2 under N-optimum conditions.

However, under N-stress condition, both the genotypes showed similar reduction in the chl-a (Fig. 2G), chl-b (Fig. 2H), total chl (Fig. 2I) and carotenoids contents (Fig. 2J).

3.2 Transcriptome Analysis

RNA sequencing of both PS2 and PS2-SRDP was carried out under N-optimum and N-stress conditions using the PacBio Sequel II sequencing platform. Raw read data for each sample are presented in Table 1. On average 3,41,535 CCS reads have been generated, with 3,31,457.625 full length non-chimeric reads and 29,010 uniquely mapped isoforms altogether.

Table 1
Analysis of Sequencing data

Sample	CCS Reads	FLNC	HQ Isoforms	Unique mapped isoforms	Unique mapped Loci
PS2CS_R1	308560	308194	39192	32526	11716
PS2CS_R2	451949	451452	49742	40070	11767
PS2NS_R1	422486	421885	45673	37323	11792
PS2NS_R2	319327	318871	37883	31135	10071
PS2CR_R1	40085	39953	4584	3269	2087
PS2CR_R2	315397	313652	44175	34246	11679
PS2NR_R1	29358	29289	3219	2202	1470
PS2NR_R2	279855	279403	38481	23257	8114
SRDPCS_R1	461077	460121	38277	31997	10702
SRDPCS_R2	574647	422544	41566	36117	13204
SRDPNS_R1	480358	479666	41490	34996	11113
SRDPNS_R2	444354	443333	41263	34581	11921
SRDPCR_R1	591110	590260	69154	53886	12949
SRDPCR_R2	53739	53617	6192	5182	3297
SRDPNR_R2	466779	466234	54997	42085	13058
SRDPNR_R1	225483	224848	30252	21293	9039
(CS: Control shoot; NS: N-stress shoot; CR: Control root; NR: N-stress root; CS: Control Shoot)					

Number of DEGs under all different comparisons is presented in Fig. 3. Differentially expressed genes under each comparison are provided in Table S2. The number of DEGs in root tissues under N-stress

conditions was higher in PS2 than in PS2-SRDP. In the case of shoot tissues, PS2-SRDP showed a greater number of DEGs in comparison to PS2 under N-stress conditions.

Root- and shoot- specific DEGs of different comparisons under N-optimum and N-stress conditions are interpreted as Venn diagram (Fig. 4). PS2-SRDP under N-stress conditions has few exclusive DEGs, which were absent in PS2-SRDP N-optimum or in PS2 under either condition. Since these genes (7 in root and 25 in shoot) are expressed only under N stress and not in control conditions, they are due to the responsiveness of the transgenic PS2 under N stress. We have studied the functional annotations of these genes using the KEGG pathway database and also included them in the KEGG and GO analyses. Among the DEGs that were specifically observed under N-optimum conditions in both PS2 and PS2-SRDP, 11 are expressed in the shoot, and none in the root, showing these DEGs may not be responsive to N-stress. Though the introduction of *OsSRDP* affected the downstream gene expressions, we did not observe *OsSRDP*-specific DEG, which might be due to the identical mRNA transcript abundance of *OsSRDP*. To confirm this, we have studied the expression of *OsSRDP* through RT-qPCR from the same set of root and shoot samples, which also shows $2^{-\Delta\Delta ct}$ value of *OsSRDP* was ≈ 1 indicating similar expression under both N-optimum and N-stress conditions (Fig. 5).

3.3 Validation of data by RT-qPCR

To validate the RNA-sequencing data, we carried out RT-qPCR analysis on a few prominent DEGs. Among the DEGs, 8 each from root and shoot data which were present in the enriched pathways were selected and checked for expression. Expression levels of the selected DEGs were presented in Fig. 6. We found that RT-qPCR data corroborated the transcriptome data.

3.4 KEGG pathway enrichment analysis

KEGG pathway analysis was performed to ascertain the DEGs in root and shoot to different plant pathways. In both root and shoot samples, DEGs specific to PS2-SRDP under N-stress (SRDPN vs SRDPC), and those are differentially expressed between PS2-SRDP and PS2 under N-stress (SRDPN vs PS2N) were selected, excluding those were common in the control condition (SRDPC vs PSC). A total of 123 DEGs in root and 109 DEGs in shoot were selected for KEGG analysis. The list of the selected DEGs with their functional annotation for KEGG analysis is provided in Table S3. Sixty-eight of the 123 DEGs chosen from the root and 96 of the 109 DEGs from the shoot had been successfully mapped to various metabolic pathways. All the DEGs of the root and shoot mapped to different metabolic pathways, and their predicted functions were provided in Table S4.

Among the various plant metabolic pathways enriched by the DEGs, 17 were likely to be common between both root and shoot. A few pathways, such as protein processing in the endoplasmic reticulum, pantothenate and CoA biosynthesis, and efferocytosis pathways, were enriched specifically by DEGs specific to the root (Fig. 7A). There were 17 other shoot-specific DEGs (Fig. 7B), which enriched different

pathways. DEGs mapped to the corresponding plant pathways in root and shoot were provided in the Fig. S1-S10 and Fig. S11-S23, respectively.

3.5 Gene Ontology Enrichment Analysis

Singular Enrichment Analysis of the same gene sets used in the KEGG analysis was performed to identify the GO terms enriched in PS2-SRDP under N-stress conditions specific to the root and shoot.

In the root, cellular components had 15 enriched GO terms, the largest number among the three classifications. SEA tree results also reveal that the identified DEGs were predominantly associated with cellular components such as cell, cell part, intracellular, intracellular organelle, intracellular part and organelle Fig. S24. In Biological process, 7 GO terms were significantly enriched (Fig. S25), suggesting that these gene sets were mainly involved in amino acid metabolism, protein synthesis and peptide production processes. In Molecular function, 8 GO terms were enriched, contributing to ribosomal structural proteins, translation factors, transport proteins and GTP-binding/GTPase proteins involved in signalling and protein synthesis Fig. S26. The Number of DEGs contributing to each enriched GO term was presented in Fig. 8A.

In the shoot, only cellular components were enriched among the selected DEGs contributing to 9 GO terms, such as cell part, cell, intracellular organelle, intracellular, organelle, cytoplasm, macromolecular complex, intracellular part and plastid Fig. S27. The number of shoot DEGs enriching these GO terms is provided in Fig. 8B.

3.6 Protein-Protein Interaction Network (PPI)

STRING network analysis suggested a functional relation between the differentially expressed genes. In the root, 27 closely connected DEGs were found to form a network, and in the case of shoot, 31 such closely connected DEGs formed another network. The resulting network from root data showed nodes with the genes *Os01g0328400*, *Os01g0752300*, *Os01g0100700*, *Os02g0284600*, *Os07g0180900*, and *Os05g0155100* having very close connections (Fig. 9A). Similarly, shoot DEGs showed close connectivity among the genes *Os05g0469800*, *Os08g0502700*, *Os01g0654500*, *Os10g0163340*, and *Os04g0677500*. (Fig. 9B). Other genes which are involving in the biological processes such as protein export, protein processing, motor proteins, photosynthesis and glutathione metabolism were also been observed to be interconnected as supporting the KEGG results.

4 Discussion

Low N-stress in higher plants causes various physiological, morphological and molecular changes. Adaptive responses such as changes in protein synthesis, primary and secondary metabolism, cellular growth, regulatory gene expression and other cellular pathways have been apparent in plants (Peng et al., 2008). Effect of *OsSRDP* gene under low N-stress has been evaluated through a comparative transcriptome of PS2 and PS2-SRDP (Fig. 10). Chronic N-stress treatment in rice seedlings for 28 days

revealed the genes that are differentially expressed effected by the overexpression of *OsSRDP*. Although the SRDP transgene itself was not identified among the significantly differentially expressed genes, this does not indicate a lack of transgene activity. The transgene expression is under the control of its promoter, which is known to respond to various abiotic stresses such as drought, salinity, and cold; however, under N-stress conditions, the gene transcript abundance may remain unchanged.

Increased root elongation is a typical adaptive response to N deficiency, allowing plants to acquire nitrogen by exploring a larger soil volume (Sun et al., 2020), which we found in the case of PS2, but PS2-SRDP maintained similar root length and biomass retention under N-stress conditions. This happened because PS2-SRDP lines were not affected much by N- stress. This is also reflected by consistently higher photosynthetic pigment levels under N-stress conditions, which significantly improved N assimilation and photosynthetic capacity in PS2-SRDP. Collectively, these morpho-physiological adaptations suggest that *OsSRDP* contributes to improved physiological performance and may enhance tolerance to N-deficient conditions; this was also supported by transcriptome data, which revealed reprogramming of plant metabolic pathways.

Number of genes were observed being differentially expressed in PS2-SRDP under N stress related to secondary metabolite and metabolic pathways. Notably, *Os03g0819600*, encoding Chalcone isomerase, a key enzyme in the flavonoid biosynthesis pathway, was downregulated. Biosynthesis of secondary metabolites and metabolic pathways were enriched under N stress conditions in wheat (Wang et al., 2019; Yan et al., 2021) and in rice, as reported previously (Zhao et al., 2018; Xin et al., 2019). Reduced expression of flavonoid biosynthetic genes may suggest that *OsSRDP* plants experience lower physiological stress despite nitrogen limitation. Secondary metabolites such as flavonoid pathways can be indirectly regulated by nutrient supply which in downstream used for defence and signalling in plants (Yang et al., 2025).

Low N availability severely affected carbon and amino acid metabolism similar to earlier reports (Sinha et al., 2018; Subudhi et al., 2020). This is because N limitation affects the glycolysis and TCA cycle-linked metabolites such as pyruvate and related enzymes. Since carbon forms the skeleton of N-containing metabolites, nitrogen and carbon metabolism are closely linked (Fataftah et al., 2018). Differential expression of genes such as Pyruvate kinase (*Os04g0677500*), Orthophosphate dikinase precursor (*Os05g0405000*), Fructose-bisphosphate aldolase 2 (*Os10g0163340*), and Glyceraldehyde-3-phosphate dehydrogenase (*Os03g0129300*), involved in Carbon metabolism, glycolysis/Gluconeogenesis, pyruvate metabolism, and amino acid biosynthesis pathways, suggests that *OsSRDP* may influence central carbon flux under N-stress. Pyruvate kinase catalyses the conversion of phosphoenolpyruvate (PEP) to pyruvate, generating ATP during glycolysis, a process central to carbon metabolism and energy production (Schormann et al., 2019). Under N limitation, plants often adjust central carbon metabolism, which influence the expression of enzymes in these pathways, even if their genes are not the primary nitrogen-signalling targets (Amoah et al., 2025).

The upregulation of the aminotransferase gene (*Os08g0502700*) in our study indicated the maintenance of amino acid interconversion and N cycling. Such regulation may enhance NUE by redistribution of available nitrogen resources under stress conditions. Amino acid metabolism as a whole, including cysteine and methionine metabolism, is often altered by nitrogen supply levels in plants (Li et al., 2025). *OS05g0135700*, coding for S-adenosylmethionine synthetase, which converts methionine into S-adenosylmethionine (SAM), a key methyl group donor involved in ethylene biosynthesis, methylation reactions, and stress signalling in plants and *OS11g0455500*, coding for adenosylhomocysteinase-like protein, which supports methyl group flux in plants under this pathway (Vizán et al., 2021), were found to be downregulated. Cysteine metabolism is dependent on glutathione synthesis, which is important for redox balance and stress responses which are frequently altered under N stress. These linked changes often show up as differential expression in amino acid metabolism networks under N deficiency. Similar metabolic changes has been reported in rice, wheat and tobacco by Li et al., (2025). When subjected to N starvation, alterations in glycolytic and amino acid metabolic pathways support survival under limited N availability (Zhao et al., 2018; Wang et al., 2019).

N deficiency often results in reduced photosynthetic efficiency and downregulation of photosynthetic genes since N is a major constituent of photosynthetic proteins. Changes in transcript abundance of photosynthesis-related genes in various other plant species in response to N availability have already been reported (Krapp, 2015; Curci et al., 2017; Fataftah et al., 2018). We have observed some gene components involved in the photosynthetic pathway being downregulated such as *Os01g0773700*-similar to the Photosystem II reaction centre W protein Fragment, *Os07g0556200*- rieske iron-sulfur protein family protein and *Os08g0435900*-similar to LHC I type IV chlorophyll-binding protein fragment. Although several photosynthesis-associated genes were downregulated in the PS2-SRDP, it maintained higher pigment concentrations under N-stress conditions. This contradiction suggests that *OsSRDP* promotes preservation of photosynthetic process functionality through a strategy beyond simple transcriptional activation of photosynthetic genes.

Under N deficiency, oxidative stress increases, and cellular antioxidant processes involving ascorbate become important (Sakhno et al., 2019). This correlated with the upregulation of *Os03g0285700*- similar to L-ascorbate peroxidase, and of *Os10g0109600*, encoding a peroxidase enzyme involved in ROS scavenging and oxidative stress defence. Genes like *Os03g0135100*, coding for glutathione S-transferase GSTF15, and *Os06g0111500*, coding for cytosolic 6-phosphogluconate dehydrogenase, were also upregulated in PS2-SRDP. The simultaneous enrichment of the glutathione and ascorbate pathways is noteworthy, as they constitute core components of the ascorbate-glutathione cycle, one of the most important antioxidant systems in plants. Maintenance of redox balance could explain the higher retention of photosynthetic pigments observed in PS2-SRDP under N stress. The enrichment of glutathione metabolism, ascorbate metabolism, and peroxidase-mediated ROS scavenging strongly suggests that *OsSRDP* participated in redox regulation during N stress.

Another important outcome of this study was the enrichment of protein processing in the endoplasmic reticulum pathway, including Sec61p (*Os03g0213100*), Calreticulin (*Os07g0246200*) and protein disulfide

isomerase-related proteins (*Os11g0199200*). While these genes are rarely reported in N-stress studies, they are known to regulate protein folding, quality control and cellular stress signalling (Lang et al., 2017; Venkatesan et al., 2021). N deficiency can alter the protein synthesis rates and induce cellular stress. Hence, modulation of the ER-associated protein-processing pathway may represent an adaptive mechanism to maintain proper protein folding under limited N conditions.

GO enrichment analysis provided added insights into the various plant processes regulated with respect to N stress. We observed that the majority of enriched GO terms belong to cellular components in both root and shoot, supported by earlier reports on rice under N stress, reflecting extensive cellular reorganisation during nutrient stress (Xin et al., 2019; Zhang et al., 2024). Protein synthesis and amino acid-related GO terms were enriched in biological processes in roots, representing an adaptive strategy to optimise N utilisation and recycling when there is restricted N supply. The enrichment of molecular function terms linked with structural constituents of ribosomes, translation factors, transport proteins, and GTP-binding proteins further supports the importance of translational regulation in the root response. Ribosome-associated genes are frequently affected under nutrient stress because protein synthesis is the most N-demanding cellular process. Modulation of this ribosomal and translational machinery may therefore help maintain cellular homeostasis by minimising metabolic cost under N stress. In contrast to root, enriched GO terms in shoot were restricted to the cellular component category. The enrichment of plastid-related components is notable because plastids are the primary site of photosynthesis, nitrogen assimilation and amino acid biosynthesis. This enrichment in PS2-SRDP shoots suggests that *OsSRDP*-mediated responses may influence chloroplast function and photosynthetic adaptation under N stress. The contrasting GO enrichment patterns between root and shoot indicate tissue-specific responses to nitrogen limitation.

Differentially expressed genes are so often functionally interconnected in the biological system. Thus, analysing their interaction provides understanding into their functional relationship (Xin and Zhang, 2020). String analysis of the differentially expressed genes suggests that they are functionally connected and participate in coordinated responses under N stress. Six DEGs that belong to the ribosomal biological pathway occupy relatively central positions and show multiple connections with other proteins in the network from the root, notably *Os01g0752300*, encoding 60S ribosomal protein L18a (RPL18A) and *Os01g0100700*, encoding 40S ribosomal protein S5 (RPS5). The presence of ribosomal subunit proteins is consistent with the enrichment of ribosome, translation and protein synthesis-related GO terms. Examining N-responsive gene co-expression networks in rice also identified genes associated with eukaryotic ribosome biogenesis (Wang et al., 2025). In shoot, strong connection was observed between the 5 DEGs primarily involved in the carbon metabolism and amino acid metabolism, *Os05g0469800*- encoding pyruvate decarboxylase, *Os01g0654500*- encoding NADP-isocitrate dehydrogenase, *Os08g0502700*, *Os10g0163340* and *Os04g0677500* functions as discussed earlier. The interconnectedness of these genes suggested a coordinated regulation of carbon flux under N-stress. Integrative transcriptomic and metabolomic analysis in rice demonstrated that N availability strongly affects carbon metabolism and genes linked with protein biosynthesis and photosynthesis

(Takehisa et al., 2015). The PPI analysis complements the GO and KEGG enrichment results and infers the interconnected nature of the molecular response towards N-stress.

Transcriptome analysis reveals changes in carbon metabolism and associated pathways such as Glycolysis/Gluconeogenesis, Ascorbate pathway, glutathione metabolism (antioxidant network) and secondary metabolites under N stress in PS2-SRDP, all of which are often modulated under N stress as plants adjust redox balance and resource allocation (Zhang et al., 2024). These coordinated metabolic adjustments are a plant adaptation to N stress, indicating that *OsSRDP* may function in multiple stress-responsive regulatory processes. Having limited information presently available regarding ER-mediated responses to N-stress in rice, these genes signify as promising targets for future functional studies. *In silico* docking and protein interaction studies may be proceeded in future to explore the underlying mechanisms of the *OsSRDP* gene under N stress, revealing previously unrecognised components of *OsSRDP* mediated stress tolerance.

5 Conclusion

OsSRDP is known for multiple stress tolerance, and we speculated N-stress response of these genes from comprehensive gene co-expression analysis using RiceFRIEND database. In our study we have found the effects the gene *OsSRDP* in the rice seedlings under chronic N stress treatment conditions through transcriptome comparison of the PS2 and PS2-SRDP lines possessing the single particular gene. Despite having the similar phenotype, we could evidently observe better photosynthetic pigment retention in PS2-SRDP under N stress. The identified set of DEGs indicated the N stress adaptation contributed by *OsSRDP* through regulation of the plant secondary metabolism, carbon-Nitrogen metabolic reprogramming and photosynthetic machinery modulation. Redox homeostasis enhancement may be a key component of *OsSRDP* mediated response towards N stress. Endoplasmic Reticulum protein processing may represent a Novel component of Nitrogen stress adaptation. GO enrichment analysis supports the metabolic reprogramming and cellular remodelling in PS2-SRDP. Interconnected PPI Network analysis provides further evidence that N-stress induces coordinated metabolic reprogramming rather than independent regulation of gene expression highlighting genes under ribosomal pathway in root and genes involving in carbon metabolism, amino acid pathway and photosynthesis in shoot forming strong networks. Over all this study indicate even the *OsSRDP* expression is negligible under N-stress but the introduction of *OsSRDP* gene itself made PS2-SRDP lines more N-resilient as compared to PS2, both morphologically as well as in gene level through the modification in the expression of downstream genes. The identified gene sets may be further studied invasive to reveal the potential interaction with *OsSRDP* and if confirmed for their role under low N metabolism, they can be utilised in the improvement of NUE in crops.

Declarations

Contributions

MS conducted the experiment, collected data, formulated the analysis, compiled results and wrote the original manuscript. KJ contributed to the tissue collection for RNA isolation and sequencing. JK and MT contributed to the RT-qPCR study. SP contributed to statistical analysis and software. AMS contributed to supervision, SRA data submission, review & editing. DCM contributed to data analysis and software. JM contributed to formal analysis, supervision, review & editing. PKM planned and designed the experiments, coordinated and supervised the entire study, acquired funding, edited and finalised the manuscript. All the authors have read and approved the final manuscript.

Consent for publication

Not applicable

Competing interests

The authors declare no competing interests

Ethical approval

Not applicable

Consent to participate

Not applicable

Funding

We acknowledge funding support from Incentivization of Research in Agriculture and the National Institute for Plant Biotechnology, Indian Council of Agricultural Research (ICAR), Department of Agricultural Research and Education, New Delhi, Government of India.

Acknowledgements

MS acknowledges the DST for awarding the INSPIRE fellowship and ICAR AICE-SRF to complete this work as part of Ph. D. thesis. The authors would like to acknowledge the Director, ICAR-National Institute for Plant Biotechnology, for the support.

Data availability

The raw data generated in this study has been available at NCBI under the BioProject ID PRJNA1525198.

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Figures

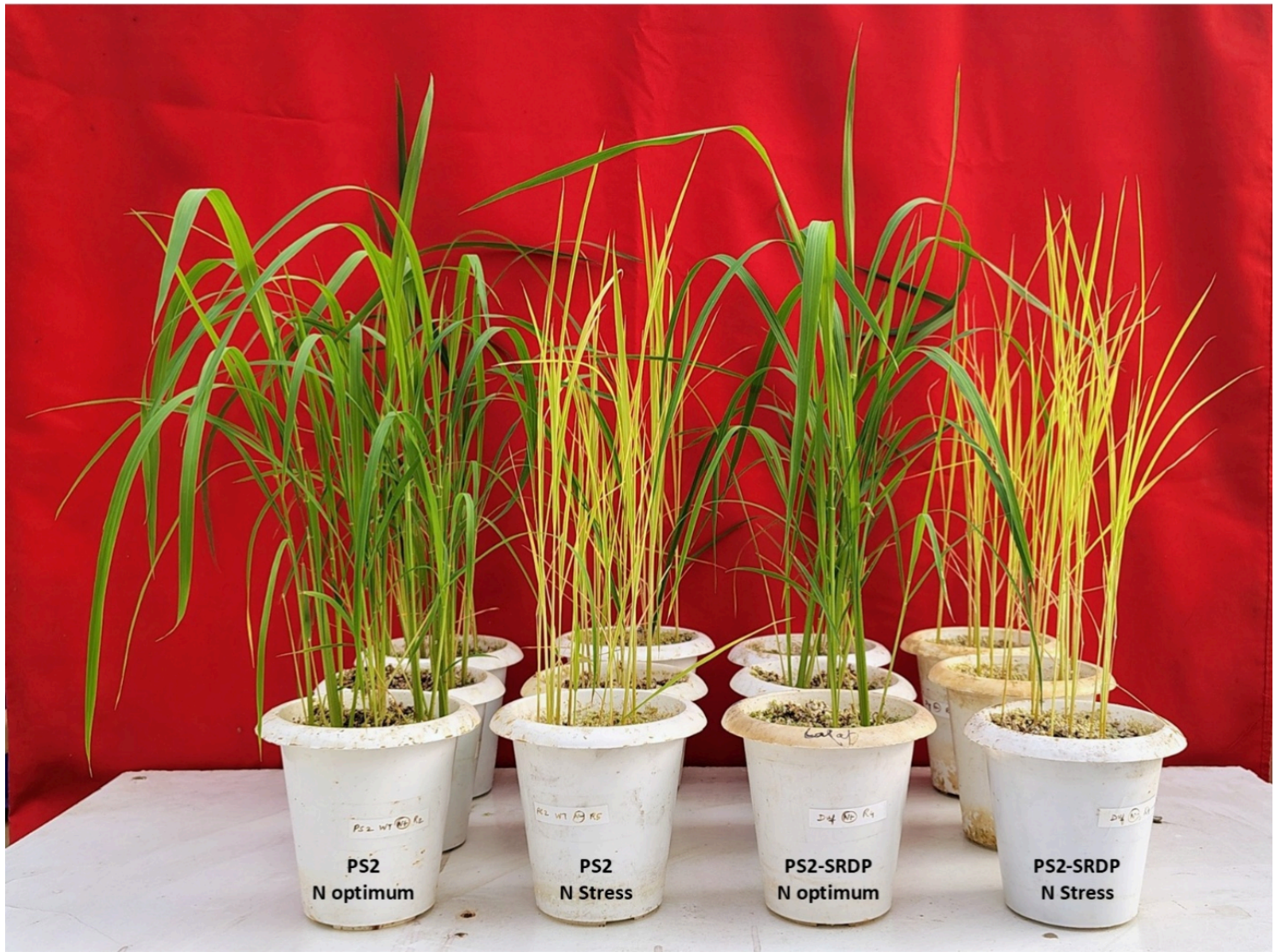


Figure 1

Wild Type (PS2) and Transgenic plant (PS2-SRDP) under N optimum and low N stress treatment after 28 days

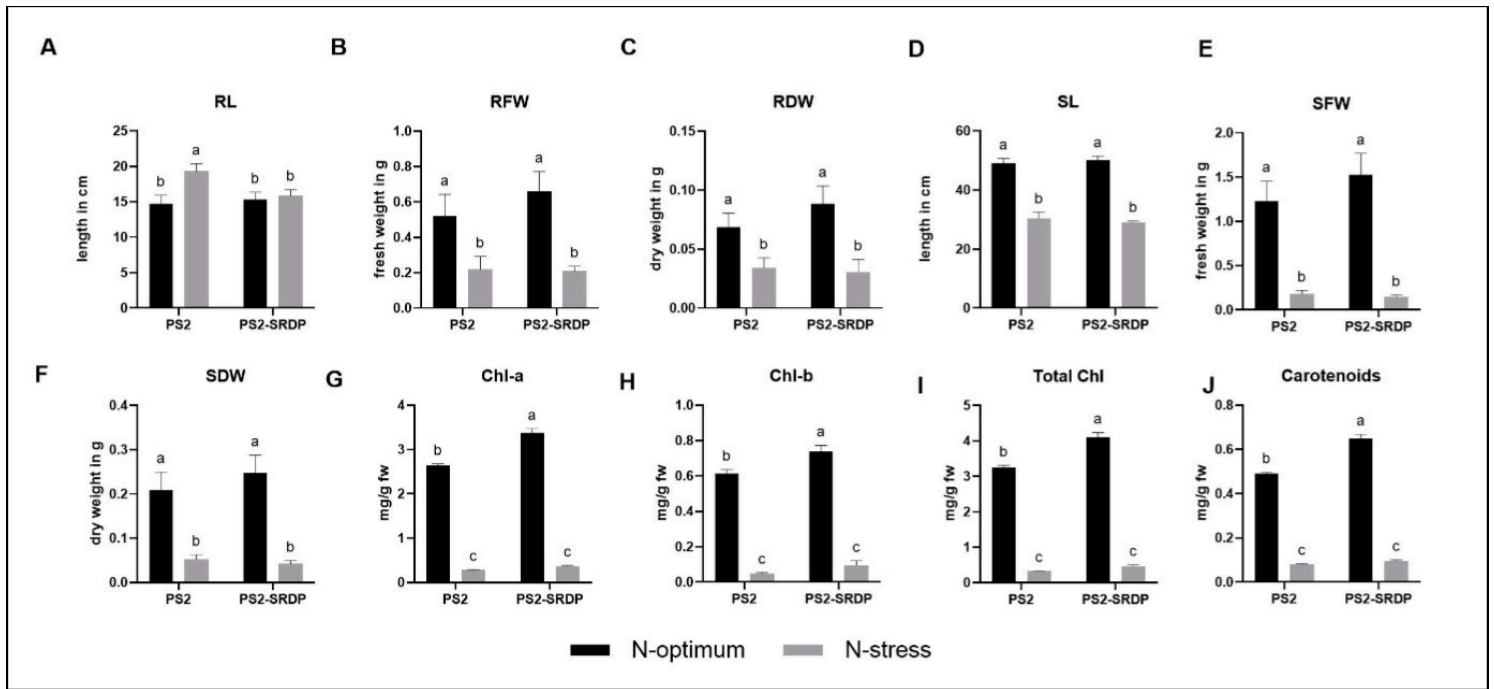


Figure 2

Morpho-physiological parameters from Root and shoot of PS2 and PS2-SRDP under N-optimum and N-stress condition (A) Root length (B) Root fresh weight (C) Root dry weight (D) Shoot length (E) Shoot fresh weight (F) Shoot dry weight (G) Chlorophyll A content (H) Chlorophyll B content (I) Total Chlorophyll content (J) Carotenoid content. The values represent the mean of three biological replicates $\pm$ SE ($n=3$). Statistically significant differences calculated using Tukey's multiple comparison test at $p < 0.05$, indicated by the lowercase letters.

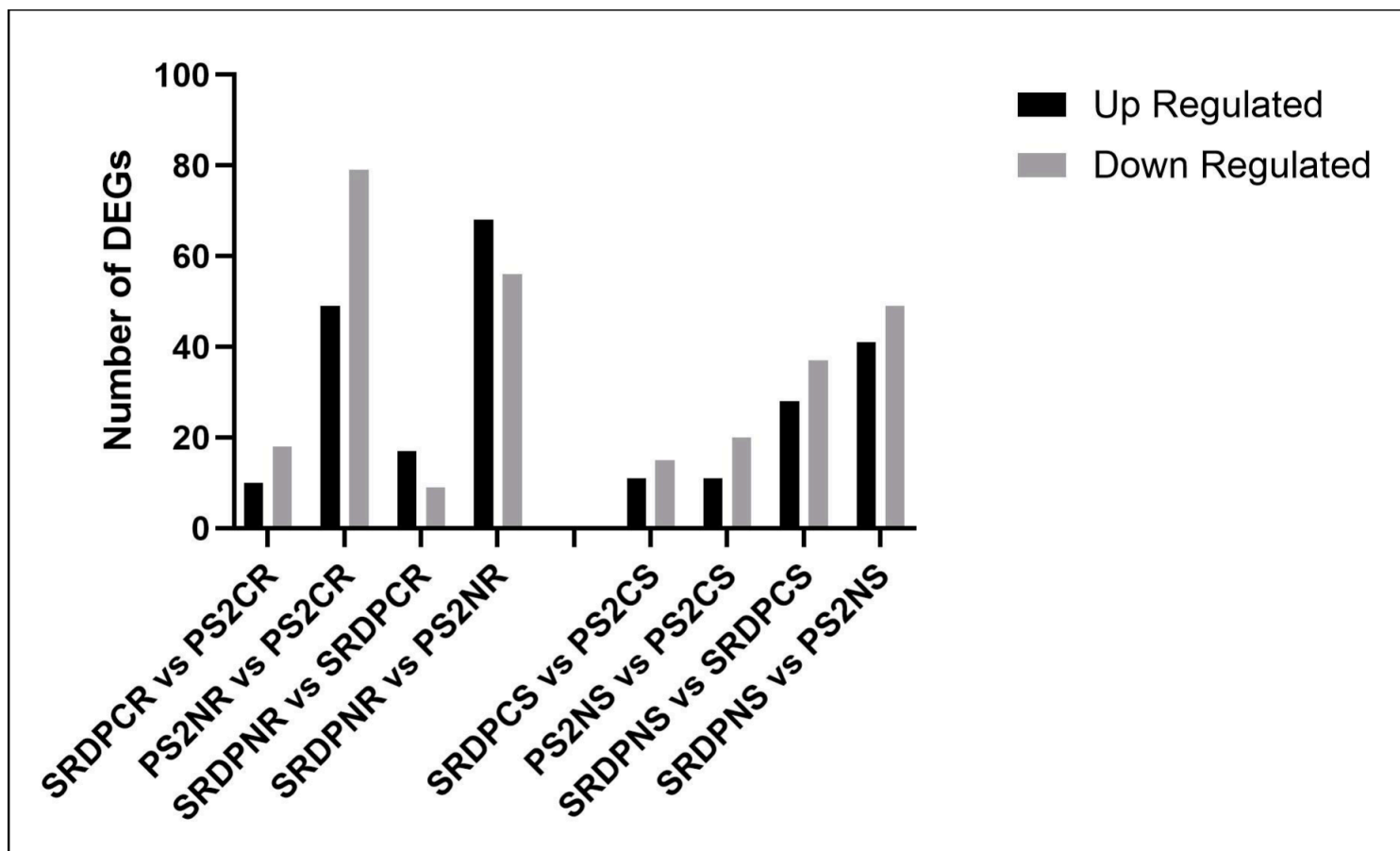


Figure 3

Bar graph showing the number of DEGs obtained from root and shoot under the four comparisons. Green and red represent the upregulated and downregulated genes, respectively

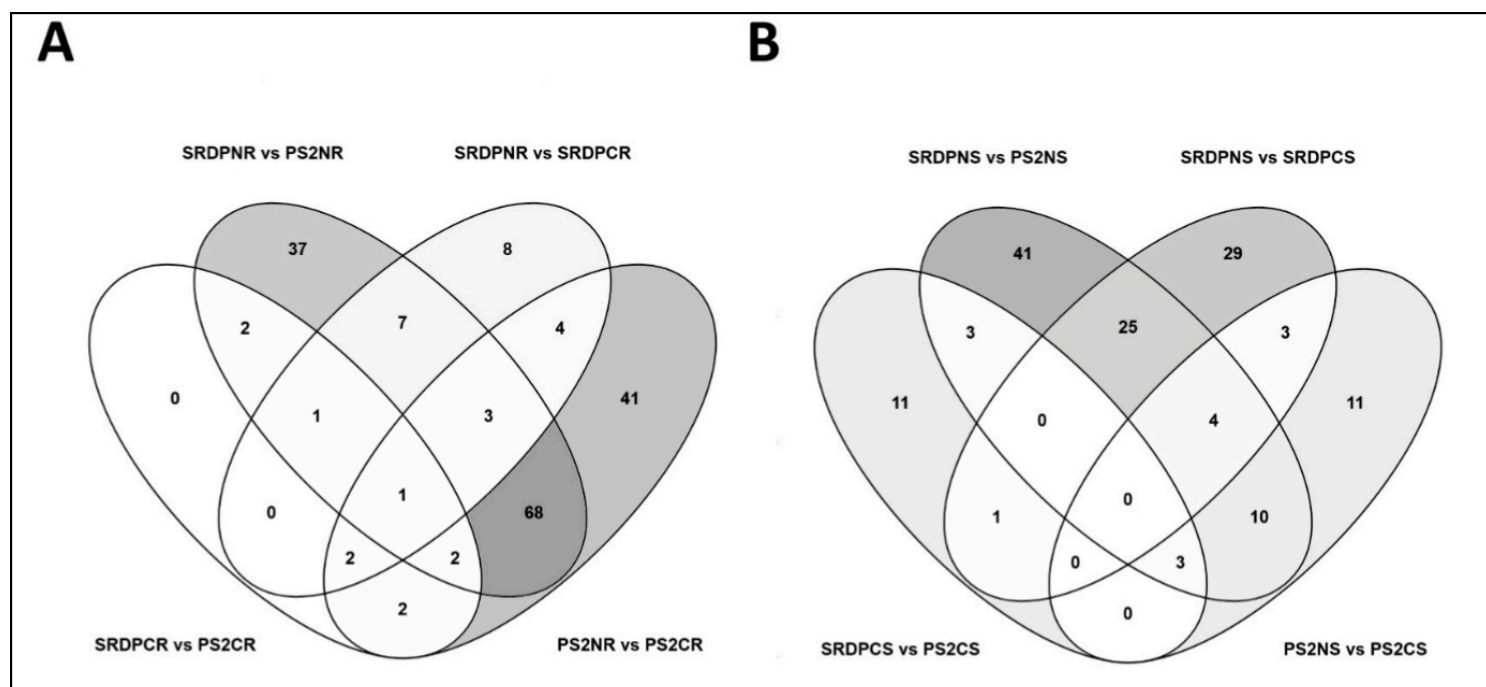


Figure 4

Venn diagram of the differentially expressed genes in Wild type (PS2) and transgenic (PS2-SRDP) under N optimum and N stress conditions: (A) DEGs from root comparisons (B) DEGs from shoot comparisons

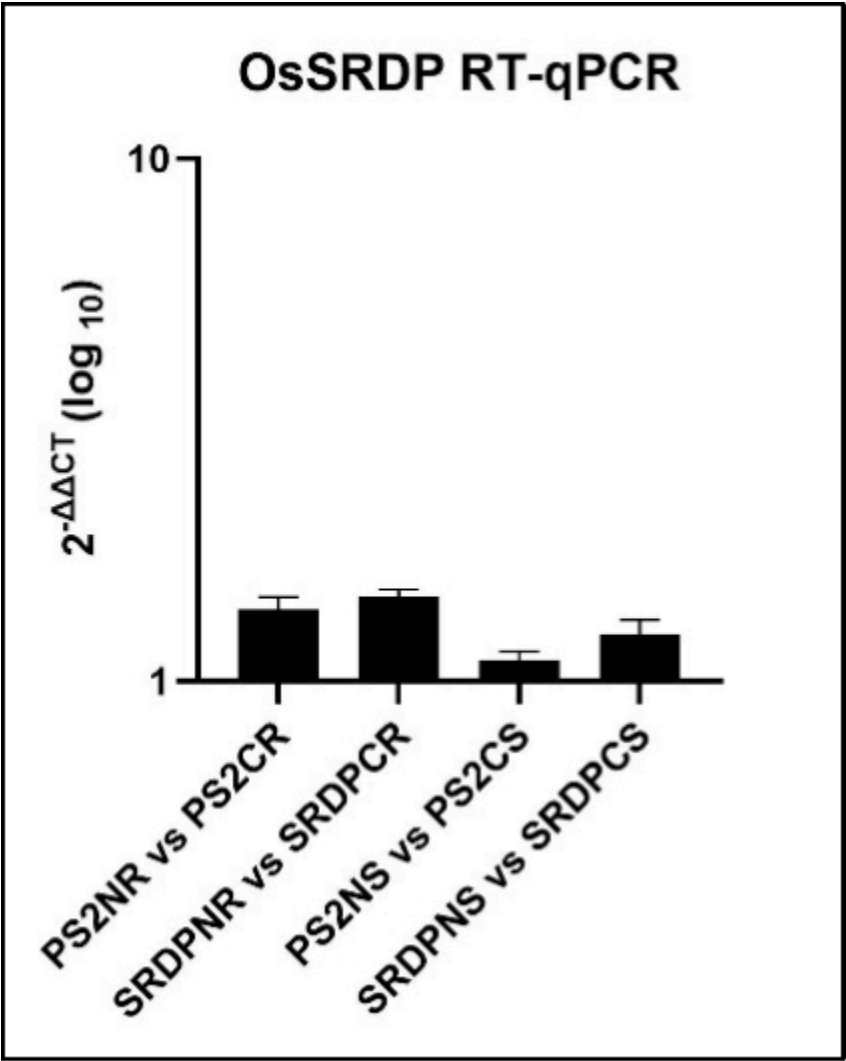


Figure 5

Expression analysis of the *OsSRDP* gene by RT-qPCR. The values represent the mean of three replicates $\pm$ SE ($n = 3$).

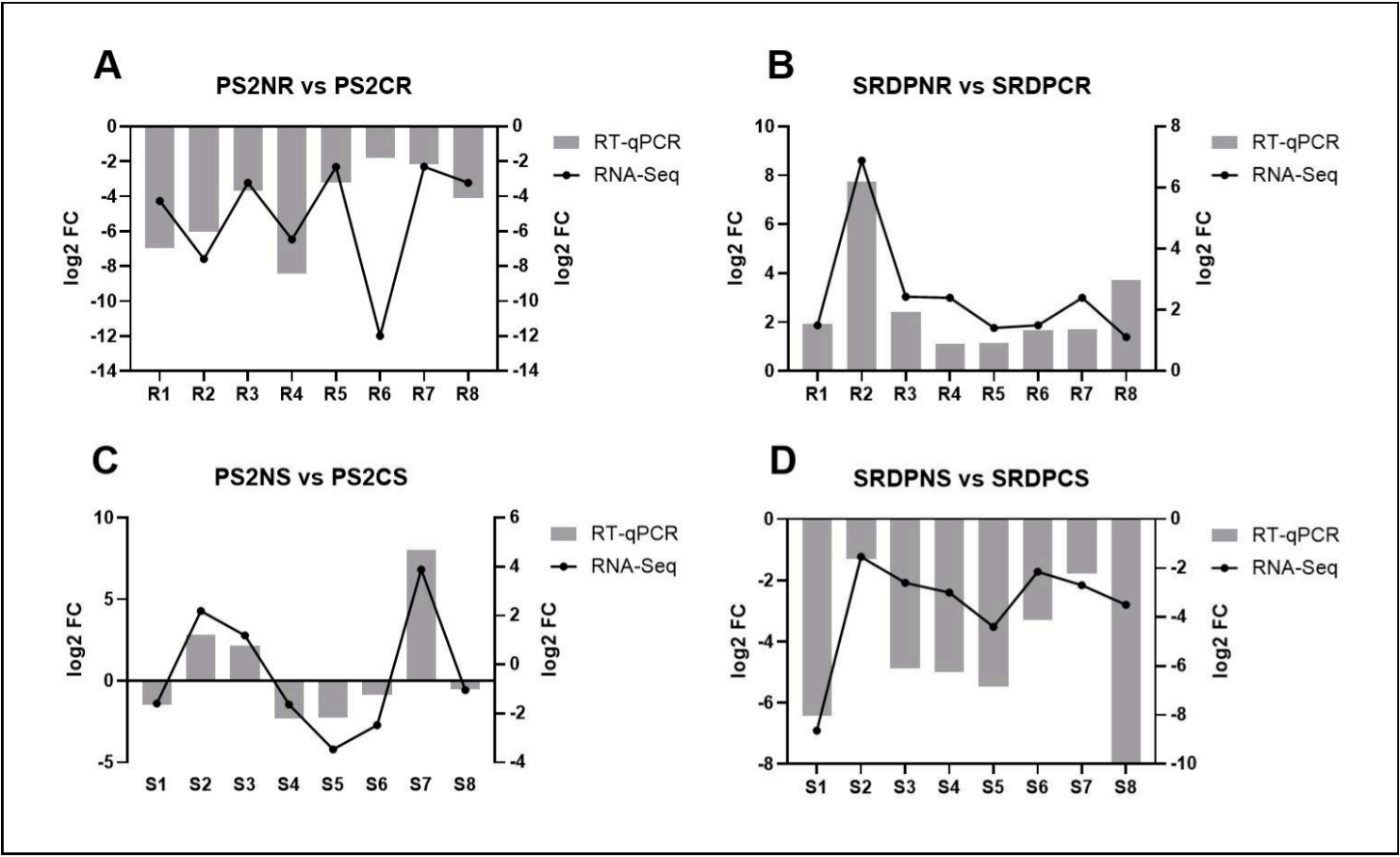


Figure 6

Validation of the selected differentially expressed genes through RT-qPCR: (A) and (B) represent the expression from root tissues, and (C) and (D) represent the expression from shoot tissues. R1-*Os03g0285700*, R2-*Os02g0605900*, R3-*Os03g0337900*, R4-*Os11g0210300*, R5-*Os07g0246200*, R6-*Os01g0752300*, R7-*Os01g0817700*, R8-*Os05g0405000*, S1-*Os03g0285700*, S2-*Os11g0455500*, S3-*Os08g0502700*, S4-*Os01g0654500*, S5-*Os08g0435900*, S6-*Os03g0129300*, S7-*Os03g0819600*, S8-*Os07g0556200*

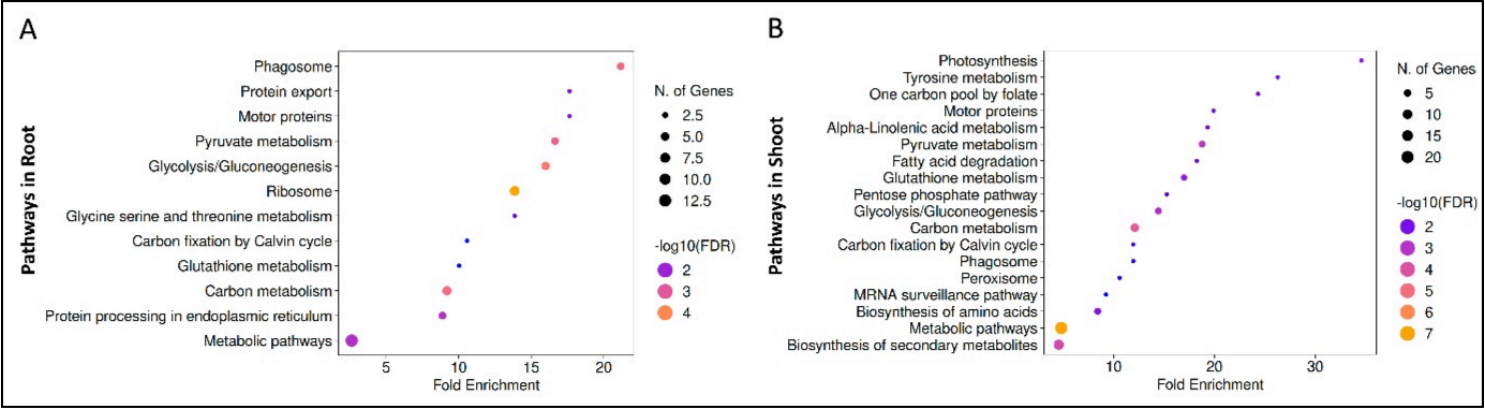


Figure 7

KEGG Pathways enriched by the Differentially expressed genes: (A) pathways enriched by DEGs in root (B) pathways enriched by DEGs in shoot

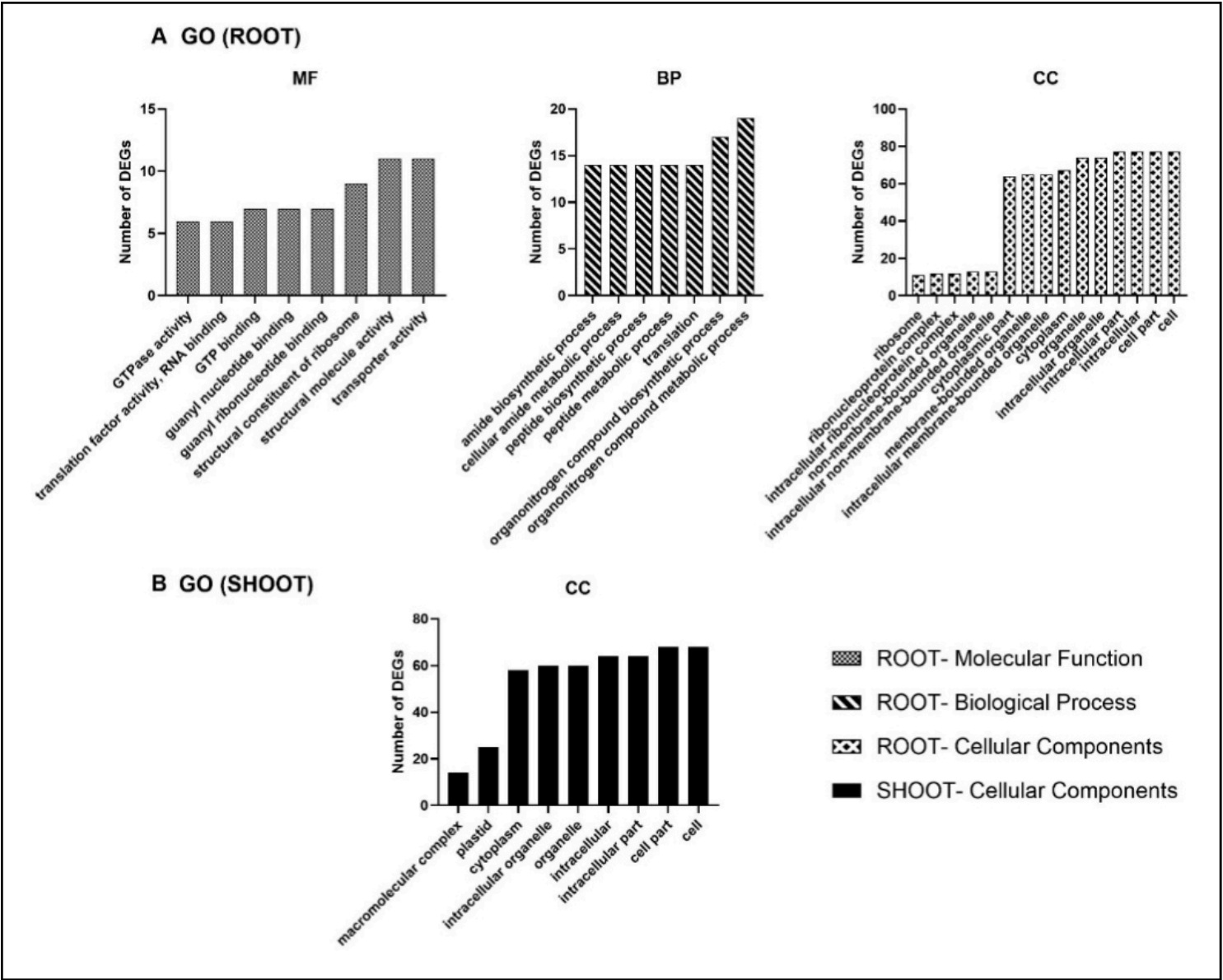
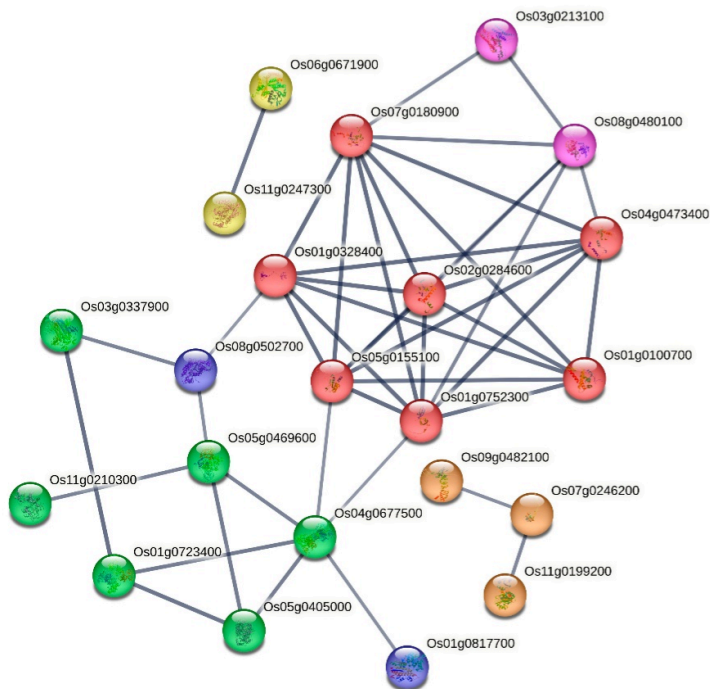
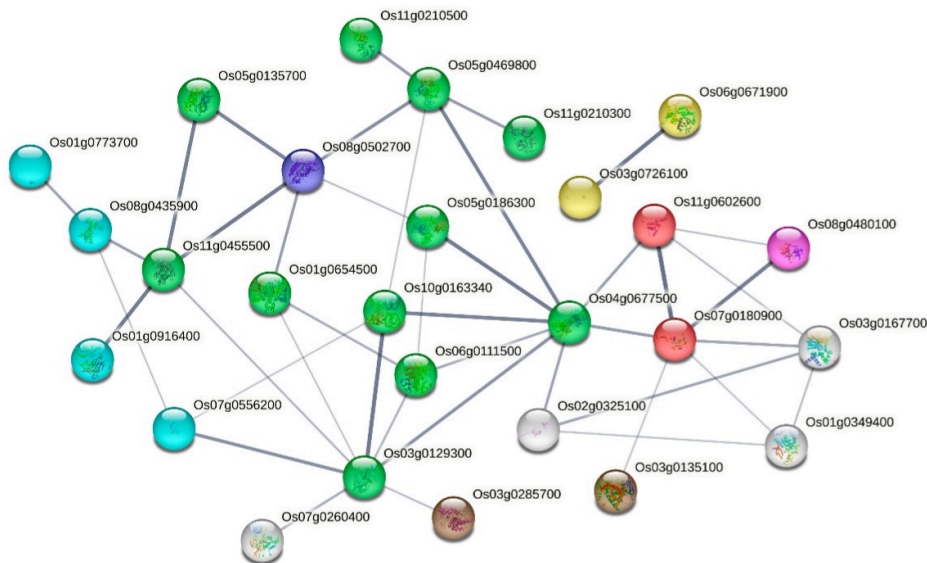


Figure 8

Functional classification of DEGs based on Gene Ontology (GO) terms: (A) GO terms enriched by DEGs in root (B) GO terms enriched by DEGs in shoot

A**PPI Network- Root****B****PPI Network- Shoot****Figure 9**

Protein -Protein Interaction Network (PPI) analysis of the differentially expressed genes from STRING: (A) ROOT DEGs (B) SHOOT DEGs; colour code represents the metabolic pathways the DEGs are involved in. Green- Carbon metabolism, Blue- Amino acid metabolism, Red- Ribosome, Violet- Protein export, Yellow- motor proteins, Orange- protein processing in ER, Turquoise- Photosynthesis, Brown- Glutathione metabolism, White- other pathways

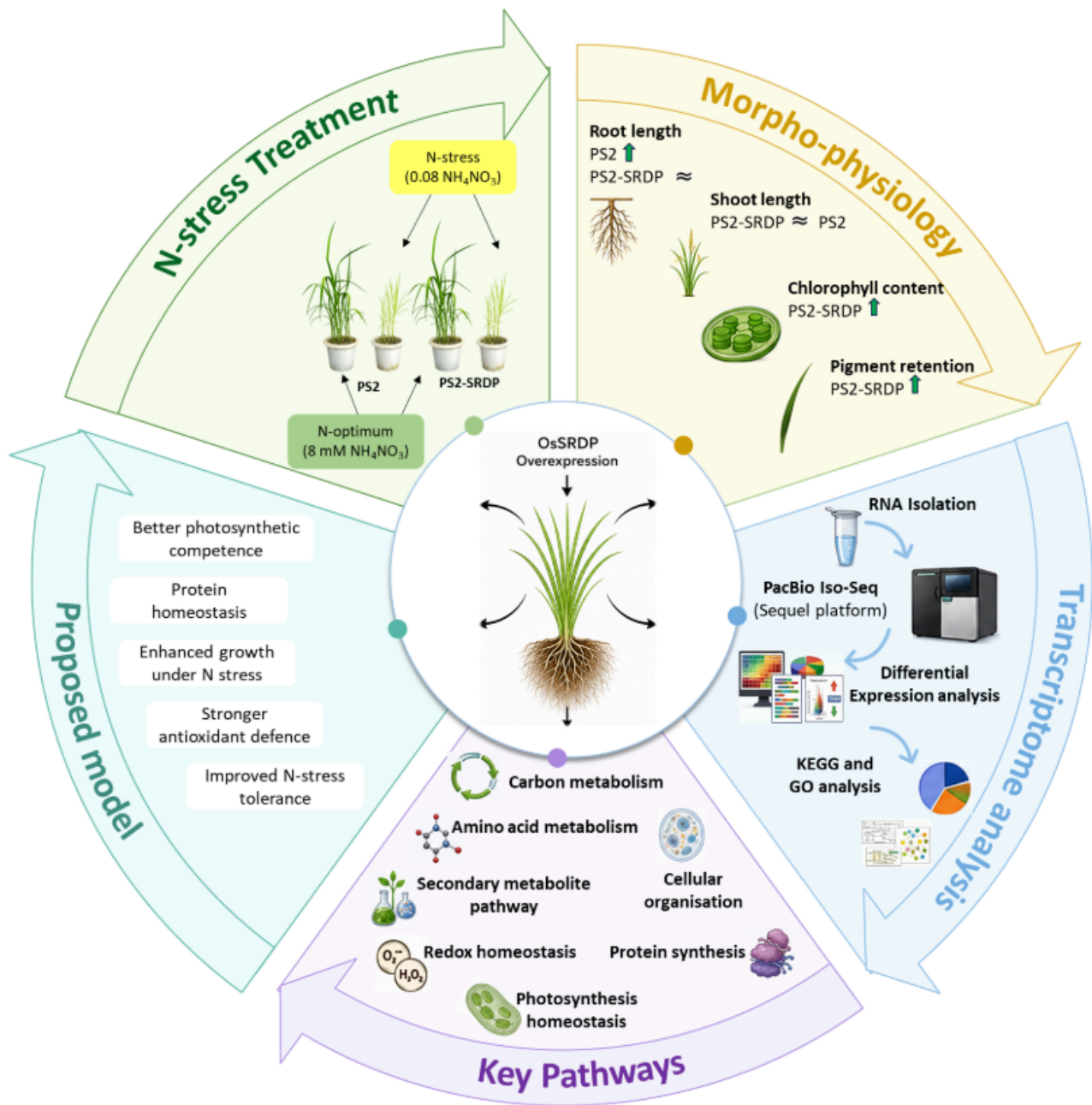


Figure 10

Summary picture of the *OsSRDP*- mediated response of rice to N-stress integrating morphological, transcriptomic and pathway level responses. *OsSRDP*- overexpressing plants PS2-SRDP and PS2 plants were evaluated under N-optimum and N-stress conditions. PacBio Iso-Seq transcriptome analysis identified differentially expressed genes and associated KEGG and GO pathways, revealing modulations in carbon and amino acid metabolism, secondary metabolism, redox homeostasis, photosynthesis, protein synthesis and cellular organisation. These responses were associated with improved pigment retention, photosynthetic competence, growth and N-stress tolerance in PS2-SRDP overexpressing plants, leading to the proposed model of enhanced physiological and molecular adaptation to N limitation.

