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ORIGINAL ARTICLE

Physio-biochemical, agronomical, and gene expression analysis reveals different responsive approach to low nitrogen in contrasting rice cultivars for nitrogen use efficiency

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Abstract

Background Nitrogen (N) is an essential macronutrient for plant growth and development as it is an essential constituent of biomolecules. Its availability directly impacts crop yield. Increased N application in crop fields has caused environmental and health problems, and decreasing nitrogen inputs are in demand to maintain crop production sustainability. Understanding the molecular mechanism of N utilization could play a crucial role in improving the nitrogen use efficiency (NUE) of crop plants.

Methods and results In the present study, the effect of low N supply on plant growth, physio-biochemical, chlorophyll fluorescence attributes, yield components, and gene expression analysis were measured at six developmental stages in rice cultivars. Two rice cultivars were grown with a supply of optimium (120 kg ha^{-1}) and low N (60 kg ha^{-1}). Cultivar Vikramarya excelled Aditya at low N supply, and exhibits enhanced plant growth, physiological efficiency, agronomic efficiency, and improved NUE due to higher N uptake and utilization at low N treatment. Moreover, plant biomass, leaf area, and photosynthetic rate were significantly higher in cv. Vikramarya than *cv.* Aditya at different growth stages, under low N treatment. In addition, enzymatic activities in cultivar Vikramarya were higher than cultivar Aditya under low nitrogen, indicating its greater potential for N metabolism. Gene expression analysis was carried out for the most important nitrogen assimilatory enzymes, such as nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS), and glutamate synthase (GOGAT). Expression levels of these genes at different growth stages were significantly higher in *cv.* Vikramarya compared to *cv.* Aditya at low N supply. Our findings suggest that improving NUE needs specific revision in N metabolism and physiological assimilation.

Conclusion Overall differences in plant growth, physiological efficiency, biochemical activities, and expression levels of N metabolism genes in N-efficient and N-inefficient rice cultivars need a specific adaptation to N metabolism. Regulatory genes may separately or in conjunction, enhance the NUE. These results provide a platform for selecting crop cultivars for nitrogen utilization efficiency at low N treatment.

Keywords Chlorophyll fluorescence · Gene expression · Leaf nitrogen content · Nitrogen use efficiency · Photosynthesis · Rice

Abbreviations

cv.	Cultivar
ETR	Electron transfer rate
F_v/F_m	Maximum photochemical quantum yield
LNC	Leaf nitrogen content
NPQ	Non-photochemical fluorescence quenching
PAR	Photosynthetically active radiation
P_n	Net photosynthesis
PSC	Photosynthesis saturation curve
PSII	Photosystem II
$_qP$	Photochemical fluorescence quenching
SP	Saturation pulse
SPAD	Soil and plant analyzer development
Y(II)	Effective photochemical quantum yield
NR	Nitrate reductase
NiR	Nitrite reductase

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GS	Glutamine synthetase
GOGAT	Glutamate synthase
C_i	Intercellar CO_2 concentration
g_s	Stomatal conductance
T_r	Transpiration rate
NUE	Nitrogen use efficiency
NupE	Nitrogen uptake efficiency
NutE	Nitrogen utilization efficiency

Introduction

Nearly half of all world's population depends on rice (*Oryza sativa* L.) for daily nutrition. There is a pressing need to increase rice grain production to alleviate world hunger in the face of a growing human population and shrinking arable land [1]. Increased nitrogen fertilizer has been employed to enhance crop yield but causing negative repercussions to human health and the ecosystem. Nearly 50–70% of all applied N is lost due to leaching, and nitrous oxide emissions from N fertilizer residues harm the atmosphere and the agricultural economy [2]. In general, crop plants do not efficiently utilize the applied N and use only 30–40% of the applied N fertilizer. Enhancing N fertilizer application may not produce a proportional enhancement in crop yields [3]. Therefore, a significant priority for a sustainable agriculture system is the development of crop plants or genotypes with high yield and N use efficiency (NUE), as well as a reduction in the amount of N applied to the soil and the amount of N lost to the environment [4]. This can be done by selecting existing cultivars or breeding new cultivars for high N use efficiency (NUE) for large-scale cropping systems.

Nitrogen supply has a significant impact on both the physiology and growth of plants. The leaves at different developmental stages regulate the physiological efficiencies of the crop plants [5]. The efficiency of photosystem II (PSII) in leaves reflects photosynthetic efficiency, which can be measured by in vivo tools [6]. Non-photochemical quenching (NPQ) measures the amount of light energy lost when more photons than needed are used in photochemical reactions during photosynthesis [7]. Photoinhibition occurs when nitrogen is scarce for rice plants because of its central function in controlling photochemical quantum yield and quenching efficiency of PSII [7, 8]. In sugar beet leaves, PSII photochemical activity and photosynthesis are negatively impacted by N deficiency, as measured by a decrease in maximum efficiency of PSII photochemistry under dark adaptation (Fv/Fm) and an increase in photochemical quenching (qP) and non-photochemical quenching (qN) [9]. In plants, photosynthesis is an essential step for plant growth and development, and its efficiency is influenced by N supply. One part of the absorbed N forms a Rubisco protein, and the second is used in other photosynthetic components [10]. To overcome the dysfunction of photosynthetic components, the remobilization of leaf N content (LNC) plays a vital role [11]. Photosystem II (PSII) is the main component of this process, which regulates electron transport flow and, thus, helps generate assimilatory powers in the form of ATP and NADPH [12].

Several studies have demonstrated that N is crucial for the regulation of leaf chlorophyll synthesis in crop plants, and that there is a strong positive correlation between the amount of N available to plants and their chlorophyll content, N content in leaves, and their ability to absorb carbon dioxide [13]. Consequently, N deficiency causes a decline in pigment system and photosynthetic efficiency, impacting carbohydrate synthesis and ultimately leading to a decline in biomass production and crop yield [14]. Many plant processes are influenced by the N status of plants, including stomatal conductance, internal CO_2, photochemical efficiency of PSII, and biochemical processes [15]. Leaf-soluble protein, of which 50% is ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), is highly responsive to N availability [16]. The energy and carbon skeletons necessary for N assimilation are produced during photosynthesis [17]. N endorses photosynthesis-related gene expression [18], while soluble sugars stimulate nitrate assimilation-related gene expression. This mutual interaction of N is crucial for plant biomass and crop yield [19].

Seed yield in relation to N inputs is defined as crop NUE [20]. Uptake of N from the soil, N translocation from root to shoot, assimilation of N in source organs (e.g., leaves or roots), and subsequent distribution (mainly as amino acids) to seed sinks are all contributors to efficient use of N for seed development [16, 21] making the process inherently complex. Moreover, during vegetative growth, plants temporarily store N in leaves and stem as nitrate, amino acids, or proteins [22]. Translocation of the stored N to seed sinks, used for growth and storage product accumulation, occurs during the reproductive stage [23]. N uptake and utilization contribute to NUE and rice cultivars from different genetic backgrounds differ in N source-sink ratio, and this process affects physiological efficiency [24].

Nitrate uptake and assimilation mechanisms have been well documented in crop plants. The uptake of nitrate and NH_4^+ by roots is achieved by specific transporters, including low and high-affinity transporters and ammonium transporters. During the vegetative stages, a considerable amount of N may be used for the metabolism of the leaf or temporary storage. In contrast, in reproductive stages, N mainly functions for the tillering and synthesizing of amino acids for grain development [25]. Leaves are the critical sources for N (re-)distribution to grains, especially flag leaves, which display high longevity concerning their metabolic activities and constitute ~ 20% of the total N content [26]. By coordinating the activities of glutamine synthetase (GS) and

glutamate synthase/glutamine-2-oxoglutarate aminotransferase (GOGAT), NH_4^+ is integrated into glutamine and glutamate, where it can then be used as an N source for the synthesis of amino acids [27]. In GS/GOGAT pathway, the two key enzymes are involved in N re-assimilation/remobilization and metabolism, as well as the biosynthesis of other amino acids, such as, cytosolic asparagine synthetase (AS) and mitochondrial NADH-glutamate dehydrogenase (GDH). The AS enzyme catalyses the conversion of glutamine to asparagine, which is a phloem transport form of N and a source of N for the biosynthesis of other amino acids in sink tissue [28]. Further, the key enzymes involved in N metabolism are the most important biochemical quality for improving NUE [16].

Detailed data analysis for the genetic basis of NUE have only been done in a few crop species. These mainly addressed root or shoot importance at the seedling or during the vegetative stage in a greenhouse or hydroponically grown plants [29, 30]. However, plant growth and productivity under controlled conditions severely differ from the field conditions [31]. Furthermore, when a plant is in its vegetative growth stage, it is primarily concerned with acquiring nutrients and storing them in its root and shoot tissues; however, when it is in its reproductive growth stage, it is primarily concerned with re-allocating resources to its flowers and grains, which requires distinct signals, molecular mechanisms, and physiological processes from those involved in vegetative growth [32, 33]. We expected that improvements in NUE and grain yield in plants would demand multifaceted acclimation, particularly in C absorption and N metabolism and transportation. It was also hypothesized that due to existing genetic variation for NUE, different rice cultivars will have different levels of physiological and agronomic efficiency. It was further predicted that rice cultivars possess physiological and agronomic efficiency differences due to existing genetic variation for NUE. Two contrasting rice cultivars with different growth and physiological efficiency were selected and grown in field conditions under optimum and low-N treatment conditions. We explored physio-biochemical, agronomic efficiency, and gene expression analysis of the two rice cultivars and identified important molecular mechanisms in growth stages that led to differences in NUE and crop yield under low-N treatment.

Materials and methods

Experimental materials and plant growth conditions

Experiments were conducted in the agriculture field of the faculty of agricultural sciences at Aligarh Muslim University, Aligarh, India (latitude 27.35° and 28.10° N, 77.29 and 78.36° E longitudes) having loamy soil with pH 6.67 in the Kharif season (June-October, 2021). Ten rice cultivars (*Panvel, Rasi, Nagina-22, Aditya, Pusa-44, Nidhi, Vikramarya, CR Dhan-310, CR Dhan-311, and Taipe-309*) were procured from the Indian Agricultural Research Institute, Pusa, New Delhi.

Seeds were sterilized with 0.1% mercuric chloride for 2–3 min and then washed five times with distilled water. Sterilized seeds were sowed in nursery beds, and 25-day-old plantlets were transplanted (at 10 × 25 cm spacing and three plants/hill were planted) in the prepared field. The plant density was 120 plants m^{-2}, and there were 40 hills m^{-2}. The field experiment (Supplementary Table S6) was based on a split plot design of plot size (2 m × 3 m) with three replicates and plots were separated by an alley 1 m wide, and the inter-varietal responses of the ten rice varieties were studied at the fifth tiller growth stage. The ten rice cultivars were screened for physio-morphological, biochemical, and chlorophyll fluorescence differences at low N treatments [60 kg h^{-1} i.e., 50% RDN (recommended dose of N)] to identify two contrasting cultivars. Rice *cv*.Vikramarya was identified as N-efficient and *cv.* Aditya as N-inefficient. *cv*. Vikramarya is semi-dwarf, long bold grains, white, resistant to GM, RTV, and GLH with an average yield of 50 Q/ha). *cv*. Aditya is semi-dwarf; grains are long bold, resistant to bold, tolerant to BS, BLB, RTV, susceptible to GM and BPH; with average yield 33–40 Q/ha. These two contrasting cultivars were further chosen for stage-specific experiments -3rd tiller, 6th tiller, flag leaf, booting, panicle, and milk stage under optimum (120 kg h^{-1} i.e., 100% RDN), and low N treatments (50% RDN) for physio-biochemical, growth, and gene expression analyses.

During the experiments, the average temperature was 25–35 °C, the humidity ranged from 52 to 57%, and the average photoperiod was 16/8 h (day/night). The average precipitation/rainfall was 112 mm to 116 mm during the crop seasons. The nutrient concentration of the field soil contained 1.02 g N kg^{-1} soil, 26.52 mg P kg^{-1} soil, and 14.27 mg S kg^{-1} soil.

Treatment details

The nutrient fertilizer was applied according to the recommended dose of N to the soil for rice growing in the study area (Supplementary Table S6). Urea was the source of applied N fertilizer (46.4% of N). Nitrogen was applied in three equal split doses (1/2 at basal, 1/3 at tillering, and 1/3 at panicle initiation stage). 50% RDN was used for the screening of ten rice cultivars with low nitrogen levels as per RDN. For stage-specific experiments, low N level (50% RDN) and optimum N level (100% RDN) at the rate of 60 kg ha^{-1} and 120 kg ha^{-1}, respectively, were applied in the stage-specific experiment. Other essential nutrients

like potassium (@40 kg ha^{-1}), phosphorus (@60 kg ha^{-1}), and zinc (@25 kg ha^{-1}) were provided in the field during the experiments. Weeding was done accordingly at 20–30 days of intervals.

Gas exchange, chlorophyll content and photochemical efficiency of PSII

Photosynthetic leaf gases attributes and relative leaf greenness were measured at the fifth fully expanded turgid leaf during the screening experiment, respectively, at low N treatment, while as 3rd tiller, sixth tiller, flag leaf, booting, panicle, and milk growth stages were used for analysis in a stage-specific experiment, respectively, at optimium and low N treatment. The photosynthetic gaseous attributes, including net photosynthetic rate (P_n), intercellular CO_2 concentration (C_i), stomatal conductance (g_s), and transpiration rate (T_r) were measured on a clear sunny day (at 9 to 11:30 am) morning using Infra-Red Gas Analyzer (CID-340, Photosynthesis system, Bio-Science, USA). The measurements were conducted under controlled conditions at ~ 390 ± 5 μmol^{-1} atmospheric CO_2 concentrations when photosynthetically active radiation (PAR) was ~ 800 µmol m^{-2} s^{-1}. A SPAD value represented relative chlorophyll content and was determined in the intact, fully expanded leaves using a SPAD chlorophyll meter (SPAD 502 DL PLUS, Spectrum Technologies, Kakamigahara, Japan) during morning hours.

A Pulse Amplitude Modulation (Mini-PAM) chlorophyll fluorometer was used to measure the chlorophyll fluorescence of PSII of rice leaves at different growth stages at low and optimium N supply (Heinz Walz, Effeltrich, Germany). Saturation pulse (SP) mode was prioritized over heat dissipation to ensure that leaves had at least 30 min to fully oxidize the PSII reaction centre before fluorescence attributes were taken using leaf clip. Maximum fluorescence yield (Fm) was produced after saturating actinic light pulse (10,000 µmol m^{-2} s^{-1} for 0.6 µs) and minimum fluorescence yield (Fo) was determined using a transmutable light (< 0.05 µmol m^{-2} s^{-1} for 1.8 µs) prior to SP different calculations were used to examine the fluorescence attributes in both the light and dark-adapted states such as (i) variable fluorescence ($F_v = F_m - F_o$) (ii) photochemical quantum efficiency of PS-II ($F_v/F_m = (F_m - F_o)/F_m$) (iii) non- photochemical quenching ($NPQ = F_m/F_{m'} - 1$) and, (iv) electron transport rate (ETR)[8] Other attributes were automatically generated with inbuilt formulated calculus by WinControl-3.29 software:

Chlorophyll fluorescence parameters	Symbols
Momentary fluorescence level	F
Illuminated minimum fluorescence level	F_o'
Illuminated maximum fluorescence level	F_m'
Maximum photochemical quantum yield	F_v/F_m
Effective photochemical quantum yield	Y(II)
Relative electron transfer rate	ETR
Coefficient of non-photochemical fluorescence quenching	*qL*
Coefficient of photochemical fluorescence quenching	*qL and qL*
Non-photochemical fluorescence quenching	*NPQ*
Quantum yield Non-regulated fluorescence quenching	*Y(NO)*
Quantum yield Non-photochemical fluorescence quenching	*Y(NPQ)*

Assessment of plant growth, yield, and its components

Plant shoot and root length were taken by metric scale from base to top in both shoot and root and represented in centimeters (cm). The plant biomass was measured after oven dried at 65 °C for 72 h and expressed as g $plant^{-1}$. Using a standard method and a correction factor (K), the leaf area was measured as follows: leaf area (cm^2) = K × length (cm) × breath (cm) whereas, range of K in rice leaves is 0.67 to 0.80, the value 0.75, is applicable for all stages of growth with the exception of the seedling stage [34].

Yield and its components was determined at the maturity stage on the randomly picked plant from the experimental field, excluding border plants for a stage-specific experiment. The yield components measured were total tillers per hill, number of panicles per hill, panicles per meter square, panicle length (cm), and grains per panicle. Grains were sun-dried before determining weight, adjusted to 14% moisture content, and represented in g 1000-$grains^{-1}$. Grain yield per meter square was calculated and represented in kg m^{-2}.

Assessment of nitrogen use efficiency

NUE, N uptake efficiency (NUpE), and N utilization efficiency (NUtE) were calculated according to Moll et al. [20]. NUpE was calculated by comparing the quantity of N applied to the amount of N found in the aboveground tillers and grains at harvest/maturity. NUtE was calculated as the ratio of seed yield to total N accumulation in the aboveground tillers, while NUE was calculated as the ratio of seed yield to total N application.

Assessment of biochemical characters

Leaf N content and enzymes activity were measured in six growth stages of both rice cultivars under optimum and low N treatment.

Analysis of leaf nitrogen content (LNC)

The plant material was rinsed thoroughly with Milli Q water, oven-dried at 65 °C for 48 h, then ground to a fine powder. A standard was prepared by using sulfanilic acid over a varied range of concentrations, and then 0.25 mg of finely ground plant material was analyzed by the Elemental analyzer (Vario EL III, CHNOS Elementar Analyzer, Germany) to measure the N content. The amount of N accumulated was measured as N content accumulation per plant and is expressed as mg/g dry weight (DW) [35].

Nitrate reductase (NR) activity

NR activity was assessed by the method of Jin et al. [36]. Briefly, Leaves from rice plants (500 mg) were frozen in liquid-N_2 and transferred in an extraction buffer of 25 mmol/l potassium phosphate (pH 7.5). The homogenate was filtered and centrifuged for 35 min at 10,000 rpm at 4 °C. The reaction mixture of enzyme extract and 100 µmol/l potassium phosphate buffer (pH 6.8) was incubated at 33 °C for 30 min, and 1 ml of 1% sulphanilamide was added to halt the reaction. Production of nitrite was estimated at 540 nm with the help of a multi-mode microplate reader (Synergy H1, Biotek Instruments Inc., Pittsburgh, PA, USA). The enzyme activity was expressed in µmol/(g fresh weight (FW) h).

Glutamine synthetase (GS) activity

The GS activity was determined according to Sun et al. [37]. Fresh leaves were homogenized in liquid-N with an extraction buffer (25 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$, β-mercaptoethanol, and 1 mM DTT). The extract was filtered and then centrifuged 10,000×*g* for 10 min at 4 °C. The enzymatic activity of the extract supernatant was measured. Sodium glutamate, $MgSO_4$, L-cysteine, and hydroxylamine were added to the enzyme extract along with a reaction mixture containing (Tris–HCl buffer (pH 8.0), ATP, and sodium glutamate). The reaction was initiated by adding hydroxylamine and incubating at 30 °C for 30 min. Using a multi-mode microplate reader, glutamyl hydroxamate (GH) concentrations were determined at 540 nm (Synergy H1, Biotek Instruments Inc., Pittsburgh, PA, USA). The activity of GS was determined using a glutamyl hydroxamate standard curve and expressed as µmol/(g FW h).

Nitrite reductase (NiR) activity

Fresh leave samples (~ 1 g) after fine powdered in liquid nitrogen were added to a 3 ml extraction buffer containing 100 mM sodium phosphate buffer (pH 8.8), 5 mM EDTA, and 1 mM cysteine-HCl and were homogenized. The homogenate was centrifuged, and the supernatant (crude enzyme solution) was used for the NiR analysis. In 2 ml reaction volume, (100 µmol Tris–HCl buffer (pH 7.5), 3 µmol $NaNO_2$, 2 µmol methyl viologen, and enzyme extract) were added. In 0.3 ml of freshly 24 µmol sodium dithionite, 0.2 M sodium bicarbonate was added to start the reaction, and the reaction was run at 30 °C for 20 min. The reduced methyl viologen's blue colour was completely removed by vigorously shaking the test tube, after which 0.1 ml aliquot of the reaction mixture was treated with 1 ml of (*w/v*) sulphanilamide (1% in 3 N HCl) and 1 ml of (0.02%) (*w/v*) N-(1-Napthyle)-ethylene-diamine dihydrochloride (NEDD). The absorbance of this mixture at 540 nm was measured and expressed as µmol NO_2^- g^{-1} FW h^{-1}.

Glutamate synthase (GOGAT) activity

The extraction buffer containing (100 mM Tris–HCl, pH 7.5, 0.2 M sucrose, 10 mM KCl, 10 mM $MgCl_2$, 10 mM EDTA, and 10 mM β-mercaptoethanol). Fresh leaf samples were homogenized in liquid- N_2 with the addition of extraction buffer filtered and centrifuged at 10,000×*g* for 10 min at 4 °C. The standard assay mixture contained 75 µmol Tris–HCl buffer, 10 µmol α-ketoglutarate, 15 µmol L-glutamine, 0.3 µmol NADH, and enzyme extract (in a final volume of 3 ml). The reaction started when NADH was added, and absorbance was measured for 3–4 min at 340 nm at room temperature. The activity of an enzyme was measured in µmol NADH oxidized g^{-1} FW h^{-1}.

RNA extraction and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from the frozen leaf at different growth stages following the manufacturer's instructions for using TRIzol reagent (Invitrogen). DNase1 (Sigma Aldrich, India) was used to eliminate any genomic DNA present in the extracted RNA. The concentration and purity of RNA was assessed using nanodrop spectrophotometer (ND1000). Using manufacturer instructions, 1 µg of total RNA was used to synthesize cDNA with a Verso cDNA kit (ThermoScientific). Rice N assimilation-related gene sequences were obtained from the online database NCBI, and gene-specific primers for qRT-PCR were designed using the online IDT PrimerQuestTool (https://www.idtdna.com) and analyzed by Oligo Analyser (Supplementary Table S1). The second stand synthesis was performed using the SYBR Green I qPCR Master mix (Thermos) on real-time PCR (light cycler 480 system (Roche diagnosis).In the light cycler following experimental conditions was used initial denaturation program (95 °C for 4 min), Amplification and quantification program (95 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min with a single fluorescence measurement) for 40 cycles, melting curve program (50–97 °C with a heating rate of 0.1 °C

per s and a constant fluorescence measurement) to verify primer specificity and final a cooling step for 10 min at a ramp rate of 1.0–2.2 °C/s. The Rice β-actin gene was used as a control to normalize gene expression values. The quantification of N assimilation-related genes was measured relative to actin using the $\Delta\Delta^{Ct}$ method and expressed as $2^{-\Delta\Delta Ct}$ [38].

Statistical analysis

Physio-biochemical, agronomic data obtained from three replicates of screening and stage-specific experiments under different N treatments were expressed as the mean ± standard error (SE). Statistical analysis for screening were analyzed by one-way ANOVA and two and three-way ANOVA for a stage-specific experiment using Originlab19b software. Pearson's correlation matrix and heatmap was performed by R studio (https://www.rstudio.com/) using the metan library, and graphics were accomplished by Originlab19b software. The significant differences between N treatments of *cv.*Vikramarya and *cv*. Aditya are indicated individually ($p^* \geq 0.05$, $p^{**} \geq 0.01$, and $p^{***} \geq 0.001$, respectively).

Results

Screening of rice varieties under low nitrogen level

Ten rice cultivars were evaluated to identify two contrasting rice cultivars at low N (N-50% RD) application. The growth performance, photosynthetic parameters, chlorophyll fluorescence, and biochemical traits were analyzed during screening at the fifth tiller stage and showed significant difference between cultivars (Supplementary Table S2, Fig. S1). The plant growth traits, such as plant height, biomass, and leaf area, were significant between the rice cultivars. The increasing trend of plant growth traits was observed in *cv.*Vikramarya, and a decline in growth performance traits was observed in *cv*. Aditya, respectively [Supplementary Fig. S2(A-D)]. The intensity of light significantly affects the chlorophyll molecules of rice cultivars, and the response of different cultivars to light-saturating intensity fluctuates under low N treatment. The highest P_n (25.11 µ mol m^{-2} s^{-1}), g_s (0.353 mol m^{-2} s^{-1}), C_i (255 µ mol CO_2 mol^{-1}), and T_r (15.64 mmol m^{-2} s^{-1}) were observed in Vikramarya whereas, the lowest response of P_n (18.68 µ mol m^{-2} s^{-1}), g_s (0.316 mol m^{-2} s^{-1}), C_i (226 µ mol CO_2 mol^{-1}), and T_r (12.53 mmol m^{-2} s^{-1}) were observed in Aditya when compared to other cultivars (Supplementary Table S2). SPAD represents relative chlorophyll content, which was highest (49.55 nmol chl. cm^{-2}) in Vikramarya and lowest (43.81 nmol chl. cm^{-2}) in Aditya rice cultivars compared to the other ten cultivars (Supplementary Table S3). The leaf nitrogen content (LNC) was also significant among the ten rice cultivars and observed higher in Vikramarya (4.60 mg g^{-1} DW) followed by Panvel and CR Dhan 310 (4.44 and 4.32 mg g^{-1} DW, respectively), and lowest LNC in Aditya (3.12 mg g^{-1} DW) (Supplementary Table S3).

The chlorophyll fluorescence efficiency of PSII showed a significant varietal difference in fluorescence attributes. The Fv/Fm, ΦPSII, and ETR were highest in Vikramarya, followed by Panvel and CR Dhan 310 rice cultivars, whereas a decrease in value was observed in *cv*. Aditya. The NPQ was higher in Aditya, Panvel, and CR Dhan 311 but lower in Vikramarya and Taipe 309 rice cultivars [Supplementary Fig. S3(A-D)]. The coefficient and yield of photochemical and non-photochemical quenching of PSII were also different among the rice cultivars (Supplementary Table S4).

Pearson's correlation, cluster, and principle component analysis of screening attributes

A Pearson's correlation heatmap displays values of the Pearson's correlation analysis, a measure of the linear strength of a relationship between two variables. The data obtained from the PCA analysis is augmented by the correlation matrix heatmap. The results of the correlation analysis between each trait of ten rice cultivars grown under low N showed that the correlation between traits reached a significant or extremely significant level (Fig. 1). Multiple groups of variables with strong positive correlations have emerged. Leaf N content ($p \geq 0.01$) showed a significant positive correlation with P_n, shoot length, biomass, g_s, C_i, *Fv/Fm, SPAD* ($r = 0.9$–0.78), chlorophyll content ($p \geq 0.001$) shows a significant positive correlation with growth attributes, leaf nitrogen content ($r = 0.94$–0.83) and negative correlation with *qL* ($r = -0.12$) and no correlation with *qP*. A strong negative correlation was detected between T_r with other gases exchange attributes $r = (-0$ to 0.47 to $-0.29)$, growth attributes ($r = -0.41$ to -0.06), *SPAD* ($r = -0.54$), *LNC* ($r = -0.62$). *Y.NO, F, NPQ, qN, Y.NPQ* ($p \geq 0.01$ to 0.001) shows a significant negative correlation with *qP* and *qL* ($r = -0.98$ to -0.54), respectively.

Principal component analysis was used to figure out the relative contribution of the different parameters to the total variation of ten rice cultivars. The results of the principal component analysis, the principal component were extracted and interpreted as different traits contributing to PC1 and PC2 during the screening of ten rice cultivars. The two principal components extracted based on the Scree plot (Fig. 2A) represent a total variance of 70.85%. The PC1 is the strongest component that contributes 45.30% of the total variance. Whereas the PC2 represents 25.55% of the total variance respectively. The parameters were categorized into four groups based on the extracted eigenvectors represented in the colour circle, which contributed to PC1

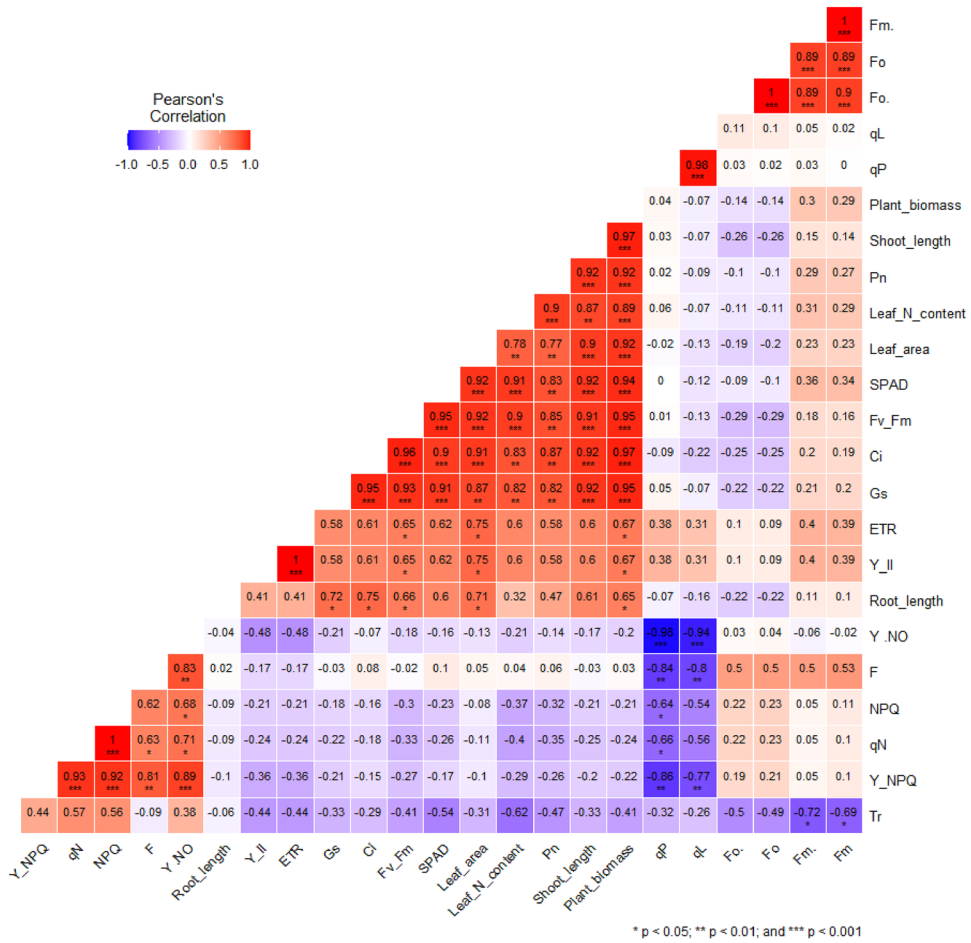

Fig. 1 Correlations between the gas-exchange, chlorophyll fluorescence, biochemical, and plant growth traits of the ten rice cultivars under the low-N supply. (+) and (−) correlations are displayed in blue and red square colours, respectively. It ranges from − 1 to + 1, whereby − 1 represents a negative linear relationship between variables, + 1 refers to a positive linear relationship between variables, and 0 shows no relationship between studied variables. The asterisks on the r-value in the figure represent the significance value ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$, respectively. (Color figure online)

and PC2 (Fig. 2B and Supplementary Table S5). The different traits in combination with multivariate analysis are successfully used to identify the most efficient cultivar (*cv*. Vikramarya) and inefficient cultivar (*cv*. Aditya) during the screening experiment.

During the screening experiment, ten rice cultivars were subjected to cluster analysis of observation for trait efficiency. Cluster analysis dendrogram based on similarity percentage (Supplementary Fig. S3) shows how the dataset of 10 rice cultivars was classified into three groups based on full linkage, correlation coefficient distance, and similarity level. Cluster 1—with blue colour represents a similarity level of 86.03% with an average distance from the centroid 64.81% (*cv*. Panvel, *cv.* Pusa 44, and *cv.* Vikramarya), cluster II—with red colour represents a similarity level of 80.79% with an average distance from centroid 99.62% (*cv*. Aditya and *cv.* Nagina 22), and cluster III—with green colour is associated with cultivars (*cv*. CR Dhan 311, *cv.* CR Dhan 310, *cv.* Taipe 309, *cv*. Rasi and, *cv*. Nidhi) respectively.

Reduction in gases exchange and SPAD under low nitrogen level

Gas-exchange attributes were calculated at six stages of growth in fully expanded leaves in two contrasting cultivars at low and optimum-N treatment (Supplementary Fig. S5). P_n in both two cultivars showed significant variation The degree of decrease of P_n in *cv*. Vikramarya was lower than Aditya, indicating *cv.* Vikramarya was less affected at low N than optimum-level of N at various growth stages, respectively. The P_n ($p \geq 0.001$) in *cv*. Vikramarya was significantly decreased at the panicle and milk stages by 12.07% and 14.37%, respectively. In *cv.* Aditya, the decline was observed from the sixth tiller to milk stages by 19.89% to 25.09%, respectively, under low N treatment compared to optimium N treatment. (Fig. 3A). The g_s was decreased significantly ($p \geq 0.002$) by 10.28% and 13.67% at panicle and milk stages, respectively, in *cv*. Vikramarya, and by 11.67%, to 18.44% at flag leaf to milk stages in *cv*. Aditya, respectively grown under low N supply than the N-100% treated plants (Fig. 3B). The significant ($p \geq 0.006$) decrease of C_i was observed at the panicle (11.19%) and milk (12.36%) stages, respectively, in *cv*. Vikramarya and by 11.37%,

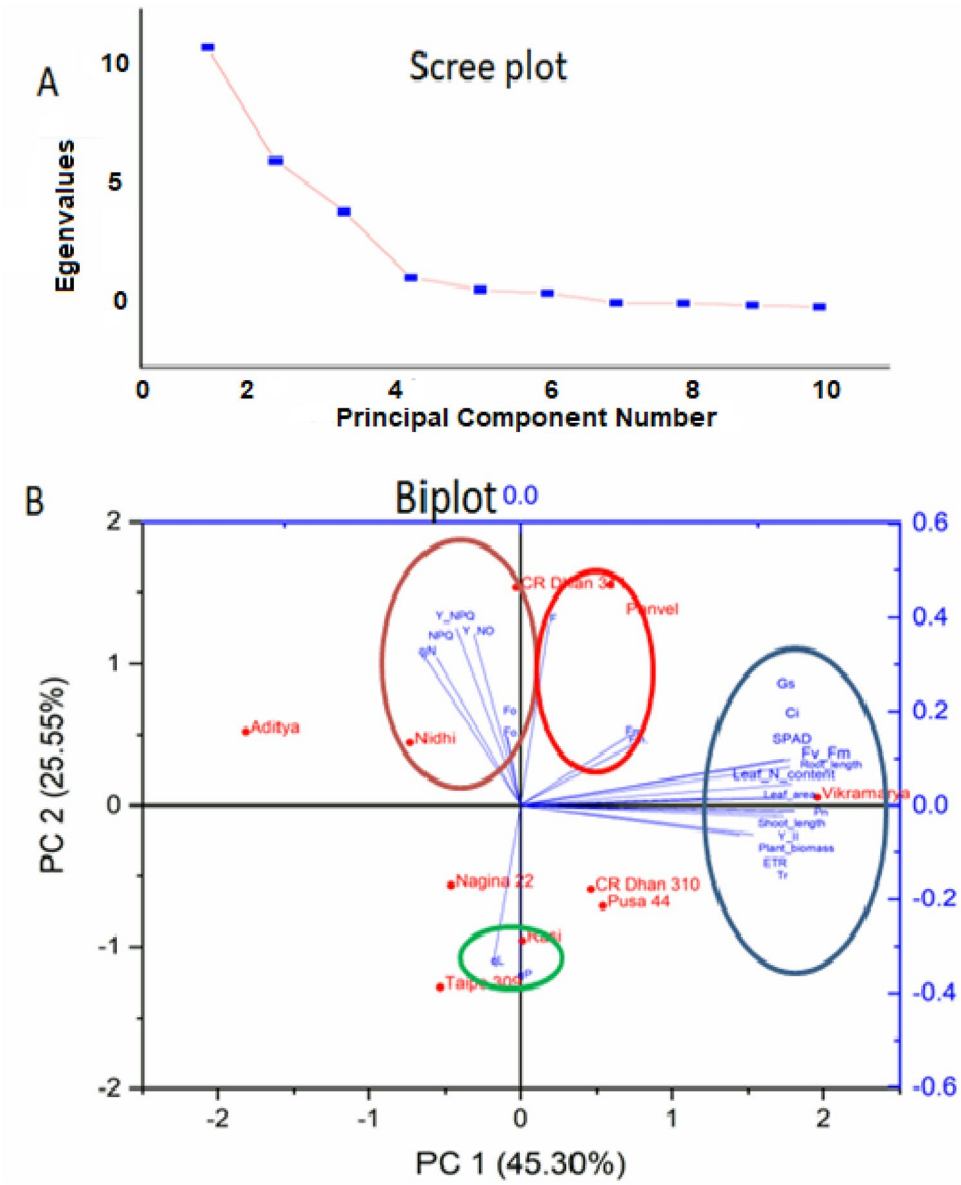

Fig. 2 Principle component analysis of growth attributes, physio-biochemical traits of ten rice cultivars under low N level **A**) Scree plot represents Eigenvalue of different attributes and, **B**) Biplot of 10 rice cultivars based on the variance in different attributes, a contrasting pair of cultivars represented as Vikramarya and Aditya

13.43%, 16.60%, and 17.53% at flag leaf, booting, panicle, and milk stages, respectively, in *cv.*Aditya under N-50% treatment than the N-100% treatment (Fig. 3C). The T_r ($p \geq 0.001$) declined significantly by 10.13% and 13.00% at panicle and milk growth stages under low N treatment in *cv.* Vikramarya and by 10.10% to 18.69% at flag leaf to milk stages in *cv.* Aditya at low N treatment than N-100% treated plants (Fig. 3D), respectively.

The SPAD value (chlorophyll content) was measured at the six growth stages of rice cultivars Vikramarya and Aditya at optimum (N-100%) and low-N level (N-50%). The SPAD values showed the relative chlorophyll content increased from the third tiller stage to the panicle stage in both cultivars under N-100% and N-50% treatment. However, there was a significant decrease of SPAD value ($p \geq 0.01$) by 11.13% and 12.66% in *cv.* Vikramarya at low N treatment. However, in *cv.* Aditya, a significant reduction of leaf greenness was observed from booting to milky growth stage by 11.32–20.71% respectively at low N (N-50%) treatment compared to respective cultivars grown at optimum N treatment (N-100%) respectively (Fig. 4).

Chlorophyll fluorescence response under low nitrogen level

Chlorophyll fluorescence efficiency Fv/Fm, $\Phi_{PSII,}$ and ETR was reduced in both cultivars grown in low N (N-50%) treatment than in cultivars grown in optimum N treatment (Table 1).In *cv.* Vikramarya, Fv/Fm was decreased significantly ($p \geq 0.001$) from booting stage (5.23%), panicle

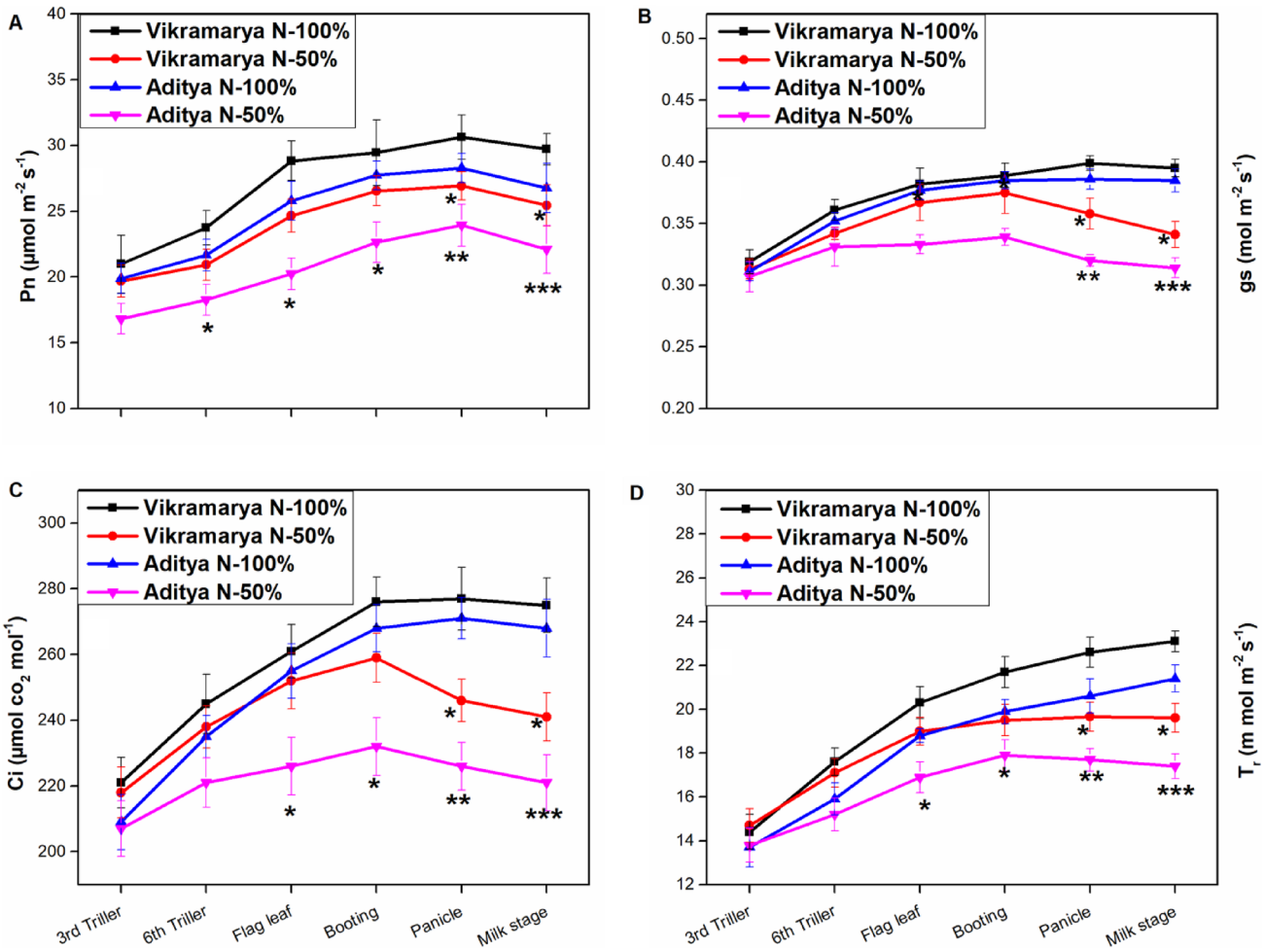

Fig. 3 Changes in gas-exchange response cultivars Vikramarya and Aditya at specific growth stages under optimum and low N treatment. P_n = net photosynthetic rate (**A**), g_s = stomatal conductance (**B**), C_i = intercellular CO_2 concentration (**C**), and T_r = transpiration rate (**D**). The data set refers to mean ± SE of each specific growth stage under optimum and low N treatment and the significant differences at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ between treatments of each cultivar represented by asterisks

stage (10.85%) and, milk stage (12.31%). While in *cv.* Aditya, there was a significant decrease in all growth stages by 7.06–18.58% at low nitrogen treatment compared to their optimum N treatment. The effective photochemical efficiency of PSII (Φ_{PSII}) was reduced significantly ($p \geq 0.001$) in *cv.* Vikramarya by about 10–11% under low N treatment. *cv.* Aditya followed the same trend from flag leaf (11.15%) to the milk stage (21.43%) grown under low N treatment than optimum treatment (N-100%), respectively. The results of the relative electron transport rate (ETR) also exhibit a similar tendency as Φ_{PSII} reduced significantly under low N treatment (N-50%) in both contrasting cultivars as compared with optimum N treatment(N-100%). The photochemical quenching coefficient (qP) and non-photochemical fluorescence quenching (NPQ) showed varied results under low N treatment compared to N-100% in both cultivars (Table 1). The qP reduced significantly by 8.03–13.68% in the flag stage to the milk stage in *cv.* Vikramarya. In contrast, the reduction in *cv.* Aditya was about 9.77–24.13% from the 6th tiller stage to the milk stage under low N level (N-50%) compared to the N-100% level. The NPQ increased upto 20–23% in *cv.* Vikramarya from panicle to milk stage whereas upto 40% increment was shown in *cv.* Aditya under low N level in all stages compared with their optimum treated cultivars, respectively (Table 1).

Effect of low nitrogen level on plant growth traits

Plant growth traits, including root length, shoot length, leaf area, and biomass, were measured in both cultivars grown in low and optimum N applications (Fig. 5). A significant difference in shoot length was observed between rice cultivars showed more plant height in *cv.* Vikramarya than

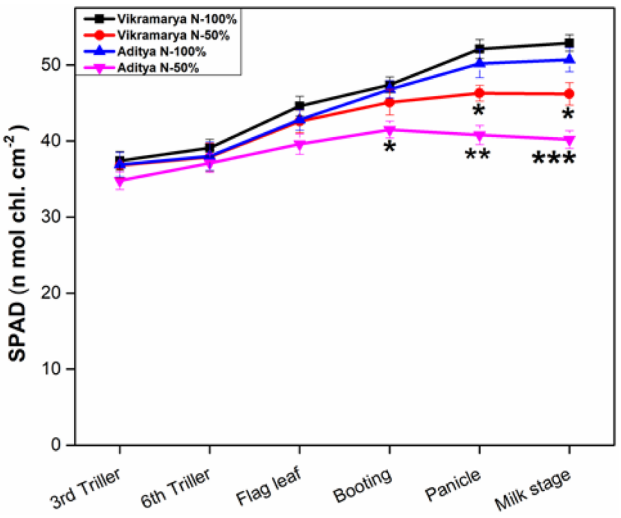

Fig. 4 Chlorophyll content decreases in rice leaves under the low-N application. SPAD = chlorophyll content in nmol chl. cm^{-2}. Data set refers to mean ± SE of each specific growth stages under optimum and low N treatment and the significant differences at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ between treatments of each cultivar represented by asterisks

Aditya in both low and optimum N treatment. Under low N treatment, *cv*. Aditya showed a significant ($p \geq 0.001$) decrease in plant height from flag stage (21.55%), booting (22.65%), panicle (23.64%), and milk stage (24.88%) than a plant grown at optimium level of N. In *cv*. Vikramarya, a significant decrease in plant height was observed at panicle (9.46%) and milk stage (11.45%) at low N level compared to their optimum level of N (Fig. 5A). Root length increased as plant growth and showed a significant increase in *cv*. Vikramarya at low N treatment at the flag (15.01%), booting (21.47%), panicle (25.55%), and milk stage while as in *cv.* Aditya at panicle (8.86%) and milk stage (10.23%) compared to an optimum level (Fig. 5B). Leaf area increased upto panicle initiation and showed a significant difference in cultivars grown under low N. In Vikramarya, leaf area decreased significantly ($p \geq 0.006$) at panicle (10.21%), and milk stage(9.80%) while as in *cv*. Aditya at low N, the reduction in leaf area was observed at flag leaf(13.17%), booting (15.46%), panicle (13.01%), and milk stage (14.16%) compared to optimum treatment (N-100%) (Fig. 5C). The biomass was found to vary in both cultivars under low N treatment. It showed a significant ($p \geq 0.005$) decrease in *cv.* Aditya at the flag (23.72%), booting (21.73%), panicle (20.80%), and milk stages (19.83%) and in *cv.* Vikramarya at the panicle (12.42%) and milk stage (12.03%) compared to optimum N treatment (Fig. 5D).

Effect of low nitrogen on yield components and N use efficiency

The results show agronomic attributes declined in both *cv*. Vikramarya and Aditya under low N treatment than the plants grown at optimium N treatment (Fig. 6). Panicle length declined in *cv*. Vikramarya and *cv.* Aditya by 8.07% and 18.22%, respectively, under low N treatment (Fig. 6A). The number of panicle per hill was declined by 13.65% in *cv.* Vikramarya and 26.22% in *cv*.Aditya respectively, and panicle per meter square by 14.01% in *cv.* And 26.22% respectively, under low N treatment than optimium N treated plants (Fig. 6B, C). In *cv*. Aditya shows more declined in grain sets in panicles (33.33%) than *cv.*Vikramarya15.47% under low N treatment (Fig. 6D). The 1000 grain weight ($p \geq 0001$) was significantly decreased in both the cultivars under low N treatment, and the decline was more observed in cv.Aditya (12.69%) than *cv*. Vikramarya 9.8% than the grains under optimium N treatment (Fig. 6E). The grain yield ($p \geq 0.000$) under low N treatment declined more in *cv*.Aditya (52.28%) than *cv*.Vikramarya (35.03%), respectively, than plants grown with optimium N treatment (Fig. 6F).

Nitrogen use efficiency showed significant improvement for *cv*. Vikramarya than *cv.* Aditya under low N treatment. Similarly, the NUpE (15.02%) and NUtE (7.58%) (Fig. 6G, H) were significantly enhanced in *cv.* Vikramarya than *cv.* Aditya under low N treatment endorses that *cv.* Vikramarya is more efficient in obtaining, allocating, and use N for grain development. Furthermore, the NUE increased in *cv*. Vikramarya by 16.78% than *cv.* Aditya under low N treatment and showed a significant difference from plants grown under optimium N treatment (Fig. 6I).

Effect of low nitrogen on the activity of N-metabolism enzymes and leaf nitrogen content (LNC)

The two contrasting rice cultivars were tested for enzymatic activity at low and optimum-N levels at six growing stages. The activities of N-assimilation enzymes (NR, NiR, GS, and GOGAT) and leaf N content showed significant variation at low N treatment compared to optimium treatment (Table 2). For NR activity, a significant decrease was observed in *cv.* Aditya from booting (30.06%), to panicle (41.53%), and milk stage (44.94%) grown under low N compared to cultivar grown under optimum-N treatment. However, in *cv*. Vikramarya, an increase in activity was observed initially at 3rd tiller, 6th tiller, flag stage, and booting stage and showed slope from booting (12.48%), panicle (28.43%), and milk stage (37.70%), respectively, under low N than cultivars grown under optimum-N treatment. Like NR activity, NiR activity also showed significant differences among cultivars at low N treatment. In *cv*. Aditya, the NiR

Table 1 Fv/Fm, Φ_{PSII} and ETR efficiencies, photochemical and non-photochemical quenching of PS II in leaves of contrasting rice cultivars at different stages of growth under optimum and low N applications

Traits	Growth stages	*cv.* Vikramarya		*cv.* Aditya	
		N-100%	N-50%	N-100%	N-50%
Fv/Fm	3rd tiller	0.798 ± 0.037	0.783 ± 0.043	0.793 ± 0.048	0.737 ± 0.032*
	6th tiller	0.811 ± 0.045	0.789 ± 0.029	0.802 ± 0.042	0.722 ± 0.040*
	Flag leaf	0.827 ± 0.038	0.797 ± 0.043	0.809 ± 0.046	0.729 ± 0.034*
	Booting	0.821 ± 0.037	0.778 ± 0.038*	0.804 ± 0.053	0.671 ± 0.044**
	Panicle	0.800 ± 0.032	0.731 ± 0.029*	0.802 ± 0.045	0.663 ± 0.056**
	Milk stage	0.82 ± 0.035	0.719 ± 0.048**	0.807 ± 0.053	0.657 ± 0.047***
ΦPSII	3rd tiller	0.686 ± 0.028	0.669 ± 0.028	0.676 ± 0.023	0.659 ± 0.028
	6th tiller	0.755 ± 0.026	0.721 ± 0.034	0.735 ± 0.022	0.653 ± 0.022**
	Flag leaf	0.764 ± 0.032	0.687 ± 0.043*	0.746 ± 0.028	0.63 ± 0.029***
	Booting	0.744 ± 0.047	0.666 ± 0.022*	0.727 ± 0.037	0.609 ± 0.033***
	Panicle	0.719 ± 0.044	0.635 ± 0.033*	0.691 ± 0.039	0.577 ± 0.030***
	Milk stage	0.678 ± 0.035	0.609 ± 0.028*	0.667 ± 0.026	0.524 ± 0.032***
ETR	3rd tiller	165.09 ± 11.1	161 ± 14.5	162.68 ± 10.1	158.59 ± 12.8
	6th tiller	181.69 ± 10.1	173.57 ± 12.2	176.88 ± 12.7	157.15 ± 11*
	Flag leaf	183.86 ± 9.7	165.33 ± 11.06*	179.53 ± 10.8	151.61 ± 11.2**
	Booting	179.04 ± 11.1	160.27 ± 13.9*	174.95 ± 12.02	146.56 ± 12.6**
	Panicle	172.03 ± 11.5	152.81 ± 11.7*	166.29 ± 13.6	138.86 ± 13.5**
	Milk stage	163.16 ± 16.00	146.56 ± 13.4*	160.52 ± 15.8	126.1 ± 12***
qP	3rd tiller	0.811 ± 0.025	0.797 ± 0.035	0.804 ± 0.042	0.781 ± 0.029
	6th tiller	0.829 ± 0.031	0.816 ± 0.038	0.818 ± 0.038	0.738 ± 0.025*
	Flag leaf	0.871 ± 0.014	0.809 ± 0.035*	0.853 ± 0.027	0.761 ± 0.041**
	Booting	0.834 ± 0.024	0.767 ± 0.023*	0.823 ± 0.034	0.729 ± 0.027**
	Panicle	0.798 ± 0.031	0.721 ± 0.032**	0.791 ± 0.035	0.689 ± 0.031***
	Milk stage	0.789 ± 0.025	0.681 ± 0.032**	0.779 ± 0.029	0.591 ± 0.049***
NPQ	3rd tiller	0.062 ± 0.010	0.073 ± 0.018	0.066 ± 0.014	0.091 ± 0.013*
	6th tiller	0.041 ± 0.018	0.055 ± 0.011	0.046 ± 0.012	0.075 ± 0.016*
	Flag leaf	0.025 ± 0.020	0.035 ± 0.020	0.031 ± 0.013	0.055 ± 0.028*
	Booting	0.048 ± 0.012	0.057 ± 0.016	0.049 ± 0.016	0.076 ± 0.017**
	Panicle	0.076 ± 0.018	0.094 ± 0.016*	0.079 ± 0.024	0.119 ± 0.023***
	Milk stage	0.097 ± 0.011	0.123 ± 0.020**	0.104 ± 0.017	0.169 ± 0.030***

Data in the table represents mean ± SE of each treatment, and the asterisks denote the significant differences at *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ between treatments of each cultivar

activity decreased with increasing growth stages from booting (13.84%), panicle (17.55%), and milk stage (20.96%). However, in *cv*. Vikramarya, a decrease was observed from panicle (17.32%), and milk stage (19.69%) under N-50% treatment of N compared to respective optimum N-100% treatment. The two cultivars showed a significant difference at all growth stages in low N treatment for GS activity. GS activity in *cv.* Aditya showed a significant decrease in flag leaf (14.90%), booting (21.47%), panicle (20.71%), and milk stage (23.49%) at low N treatment compared to optimium treatment. *cv.* Vikramarya showed a significant decrease in GS enzyme activity at panicle (17.44%) and milk stage (28.90%) grown at low N treatment compared to N-100% N treatment. The GOGAT activity in both cultivars under low N treatment initially increased and then decreased with the growth stages. In *cv*. Vikramarya, the decrease in activity ranges from the booting stage (21.05%), panicle (25.64%), and milk stage (28.16%). Similarly, *cv*. Aditya also showed a significant decrease at booting (27.94%), panicle (36.61%), and milk stage (44.78%) compared to respective optimum N-100% treatment. Leaf N content in leaves increased in both cultivars when the N treatment was optimium. However, it decreased in low N-treated cultivars as the plant matures. Under low N treatment, a significant ($p \geq 0.001$) decline was found in *cv.* Aditya at the flag (11.24%), booting (20.43%), panicle (25.58%), and milk stages (31.52%). Similarly, *cv*. Vikramarya showed a significant decrease of LNC in panicle (10.18%) and milk stages (13.98%) at low N treatment, respectively, compared to optimum-N treatment.

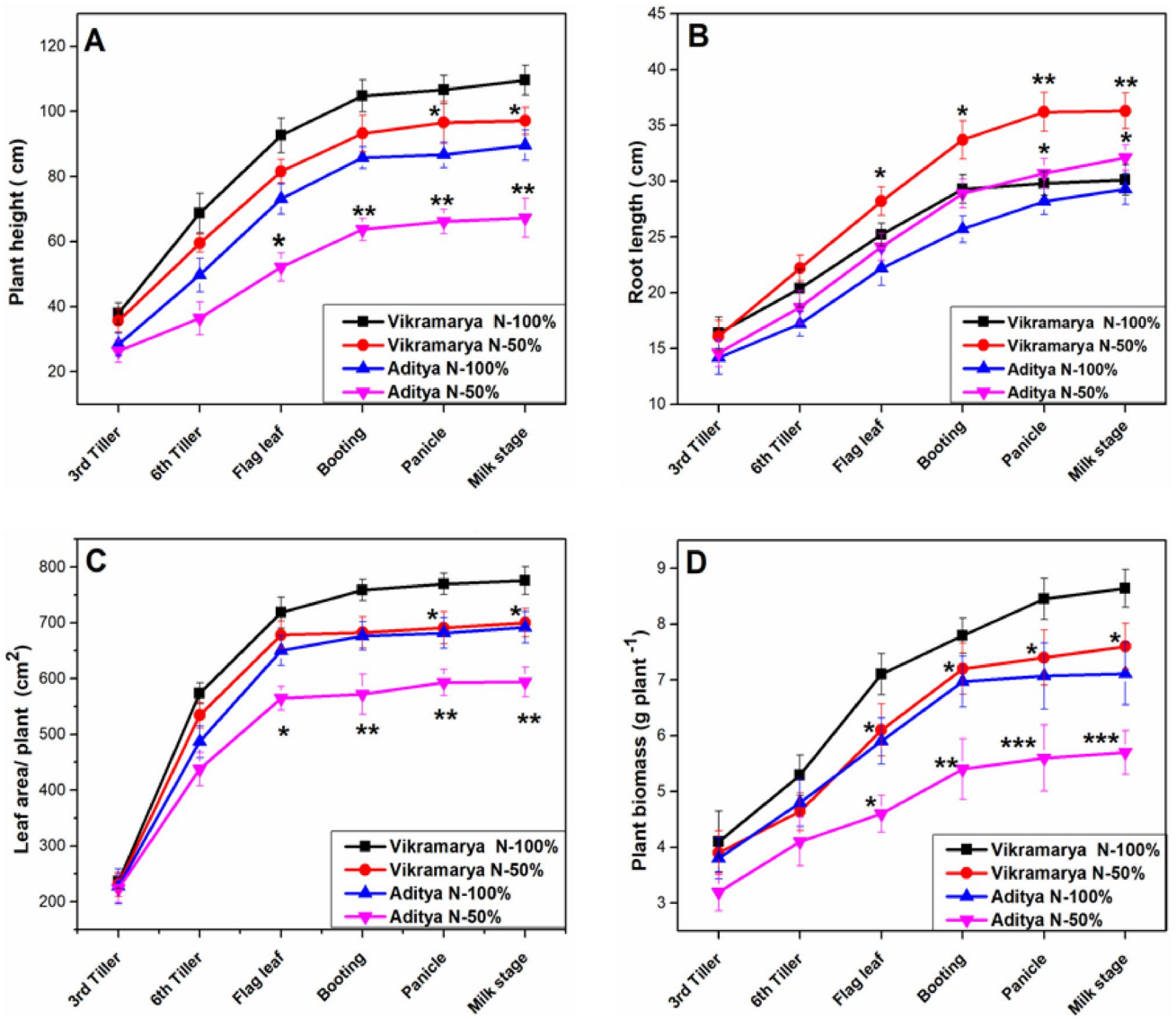

Fig. 5 Variation in plant growth of rice cultivars Vikramarya and Aditya at six growth stages under low and optimum N applications. Plant height (**A**), root length (**B**), leaf area per plant (**C**), and plant biomass (**D**). Data set refers to mean ± SE of each specific growth stages under optimum and low N treatment and the significant differences at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ between treatments of each cultivar represented by asterisks

Expression pattern of NR, NiR, GS, GOGAT in rice cultivars at low N treatment

To find out low N affects the expression of key genes involved in nitrogen metabolism, RT-qPCR was used to compare the expression levels of genes encoding NR (nitrate reductase), NIR (nitrite reductase), GS (glutamine synthetase), and GOGAT (glutamate synthase) in the leaves at different growth stages in two contrasting *cv*.Vikramarya and *cv*.Aditya under low and optimum N- treatment were analyzed by RT-qPCR (Supplementary Fig. S6). The relative expression of gene NR increases significantly from 3rd tiller to milk stage (3.22–7.6 folds)) in *cv*. Vikramarya. In *cv*. Aditya, the highest expression level was observed in the booting to milk stage (4.35–4.43 folds) under N-50% treatment compared to the N-100% treatment (Supplementary Fig. S6A). The expression level of NiR were significantly higher in all growth stages and showed an increase in expression level from 3rd tiller to panicle stage (1.94–4.62 folds) and then a decline in milk stage (0.52 folds) in *cv*.Vikramarya under low N. Similarly, in *cv*. Aditya, the expression of this gene was significantly higher from flag leaf to milk (2.49–3.62 folds) stage but lower than compared to *cv*.Vikramarya at low N treatment compared to respective optimum treatment of N (Supplementary Fig. S6B). The expression level of GS1.1 in *cv*.Vikramarya was highly

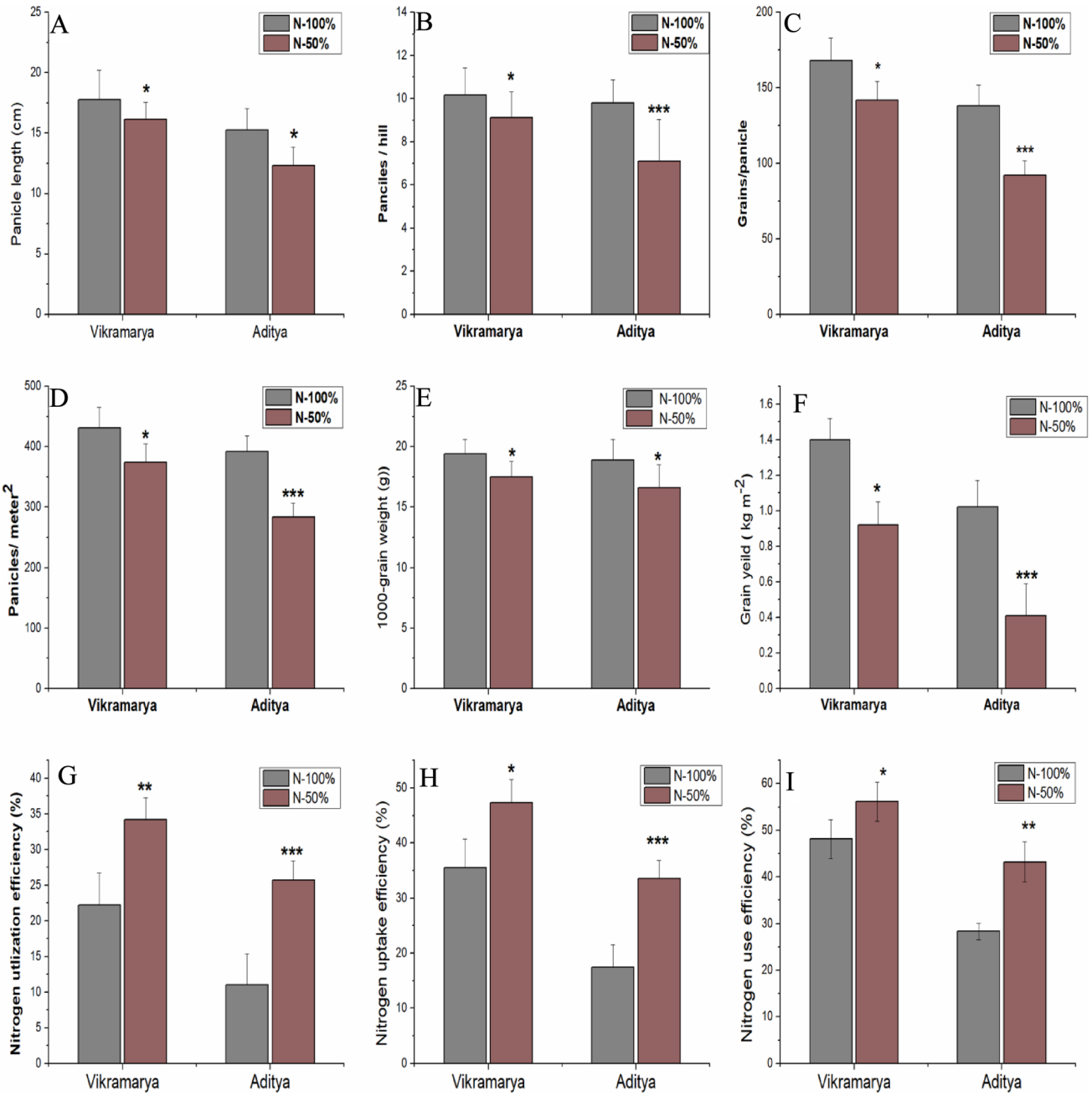

Fig. 6 Yield components and Nitrogen use efficiency of *cv.* Vikramarya and *cv.* Aditya under optimium and low N treatment. Panicle length in centimeters (**A**), number of panicles per hill (**B**), Number of grains per panicle (**C**), Number of panicles per meter square (**D**), 1000 grain weight in gram (**E**), grain yield per meter square (**F**), N utilization efficiency (NUtE) (**G**), N uptake efficiency (NUpE) (**H**), and N use efficiency (NUE) (**I**). Bars in each graph represent the mean ± SE of each treatment, and the asterisks denote the significant differences at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ between treatments of each cultivar

significant in all growth stages, from 3rd tiller to milk stage (2.14–3.99 folds). In *cv*. Aditya, the expression level was significant and increased from 3rd tiller to flag, and booting stage (1.42–3.10 folds) and remained constant to milk stage but was lower than the expression of *cv*.Vikramarya grown at low N treatment (Supplementary Fig. S6C). The expression level of GS1.2 was significantly higher from flag leaf to milk stage (4.26–5.82 folds) in *cv*.Vikramarya, whereas the same trend in expression level were observed in *cv*.Aditya from flag leaf to milk stage (3.41–3.96 folds) showed lower expression level compared to *cv*.Vikramarya at N-50% treatment compared to N-100% in both cultivars (Supplementary Fig. S6D). The relative expression level of GS2 was increased after every growth stage from 3rd tiller to milk

Table 2 N-assimilation enzyme activities and leaf N content in rice cultivars Vikramarya and Aditya at six growth stages under optimum and low N treatments

Traits	Cultivar	Treatment	3rd tiller	6th tiller	Flag leaf	Booting	Panicle	Milk stage
NR (μmol^{-1} g^{-1} FW h^{-1})	Vikramarya	N-100%	6.52 ± 0.57	6.82 ± 0.35	7.89 ± 0.42	8.81 ± 0.34	9.32 ± 0.65	9.18 ± 0.62
		N-50%	5.13 ± 0.48	5.57 ± 0.36	6.52 ± 0.52	7.71 ± 0.51	6.67 ± 0.41*	5.72 ± 0.39*
	Aditya	N-100%	4.12 ± 0.43	4.91 ± 0.53	5.87 ± 0.61	7.55 ± 0.52	7.03 ± 0.45	6.32 ± 0.41
		N-50%	3.81 ± 0.39	4.29 ± 0.45	5.06 ± 0.53	5.28 ± 0.49*	4.11 ± 0.43*	3.48 ± 0.52**
NiR activity (μmol g^{-1} FW h^{-1})	Vikramarya	N-100%	33.21 ± 2.12	35.55 ± 2.02	39.14 ± 2.01	42.51 ± 2.97	40.01 ± 2.73	37.09 ± 2.74
		N-50%	31.61 ± 2.17	32.83 ± 2.34	36.11 ± 2.62	38.62 ± 2.34	33.08 ± 2.12*	29.79 ± 2.22**
	Aditya	N-100%	26.14 ± 2.62	30.14 ± 2.31	33.41 ± 0.34	36.32 ± 2.49	35.31 ± 2.87	32.63 ± 2.53
		N-50%	24.73 ± 2.41	29.3 ± 2.21	31.78 ± 2.34	31.29 ± 2.65*	29.11 ± 2.84*	25.79 ± 2.41**
GS activity (μmol g^{-1} FW h^{-1})	Vikramarya	N-100%	1.29 ± 0.052	1.42 ± 0.067	1.59 ± 0.075	1.69 ± 0.068	1.72 ± 0.067	1.73 ± 0.043
		N-50%	1.21 ± 0.062	1.37 ± 0.057	1.47 ± 0.053	1.56 ± 0.049	1.42 ± 0.034*	1.23 ± 0.043*
	Aditya	N-100%	1.19 ± 0.056	1.32 ± 0.067	1.41 ± 0.068	1.49 ± 0.061	1.4 ± 0.0532	1.32 ± 0.043
		N-50%	1.17 ± 0.049	1.24 ± 0.057	1.2 ± 0.061*	1.17 ± 0.053*	1.11 ± 0.0432**	1.01 ± 0.037***
NADH-GOGAT (μmol g^{-1} FW h^{-1})	Vikramarya	N-100%	0.53 ± 0.043	0.59 ± 0.053	0.68 ± 0.043	0.76 ± 0.034	0.78 ± 0.032	0.71 ± 0.047
		N-50%	0.51 ± 0.051	0.55 ± 0.052	0.62 ± 0.062	0.61 ± 0.057	0.58 ± 0.043*	0.51 ± 0.053*
	Aditya	N-100%	0.47 ± 0.040	0.51 ± 0.052	0.59 ± 0.058	0.68 ± 0.044	0.71 ± 0.063	0.67 ± 0.060
		N-50%	0.44 ± 0.053	0.48 ± 0.045	0.58 ± 0.063	0.49 ± 0.035*	0.45 ± 0.066**	0.37 ± 0.051**
Leaf nitrogen content (LNC) (mg g^{-1} DW)	Vikramarya	N-100%	4.10 ± 0.142	4.90 ± 0.137	5.31 ± 0.104	5.46 ± 0.150	5.5 ± 0.193	5.59 ± 0.183
		N-50%	3.90 ± 0.171	4.70 ± 0.163	5.20 ± 0.188	5.00 ± 0.145	4.94 ± 0.121*	4.81 ± 0.150*
	Aditya	N-100%	3.25 ± 0.198	3.55 ± 0.186	4.27 ± 0.179	4.55 ± 0.195	4.69 ± 0.180	4.79 ± 0.167
		N-50%	3.19 ± 0.177	3.49 ± 0.199	3.79 ± 0.152*	3.62 ± 0.166*	3.49 ± 0.197**	3.28 ± 0.202***

Data in the table represents mean ± SE of each treatment and the significant differences at *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ between treatments of each cultivar denoted by asterisks

stage (3.21–4.69 folds) but shows a significant increase in their expression level from flag leaf to milk stage under low N treatment in both cultivars. In *cv*.Vikramarya, increase in the expression level of GS2 from 3rd tiller, 6th tiller, and flag leaf and then remain stable from booting to milk stage. In *cv*. Aditya, a significant increase in expression level was observed from flag leaf to milk stage (2.94–3.76 folds) but lower than *cv*.Vikramarya at low N treatment (Supplementary Fig. S6E).

The expression level Fd-GOGAT1 in *cv.* Vikramarya were highly significant from 6th tiller to the milk stage (4.80–6.45 folds) and showed increase in expression with growth stages at low N treatment. However, in *cv.* Aditya a significant increase in expression was observed from flag leaf to milk stage (3.25–4.50 folds). At low N treatment, the expression of enzyme Fd-GOGAT2 in *cv.* Vikramarya increased from 3rd tiller to the booting stage (2.89–4.69 folds) and then remained stable upto milk stage. Similarly, in Aditya, the maximum expression levels of this genes were detected at flag leaf, booting, panicle, and milk stages (2.74–3.64 folds) under low N treatment (Supplementary Fig. S6F-G). The transcript level of NADH-GOGAT1 in *cv*. Vikramarya was highly significant with growth stages and increased from 3rd tiller to milk stage (2.54–4.46 folds). Interestingly, in *cv.* Aditya, the highest expression levels were observed from 6th tiller, flag leaf, and booting stage to milk stage (2.05–3.93 folds) grown at low N treatment (Supplementary Fig. S6H). In contrast, the expression level of NADH-GOGAT2 was greatly significant in *cv.* Vikramarya 6th tiller, flag leaf, booting stage, panicle, and milk stages (1.20–5.39 folds). Similarly, the expression level in *cv.* Aditya showed significant expression from flag leaf to milk stage (2.14–3.96 folds) and remained almost stable compared to *cv.* Vikramarya grown at low N treatments in comparison to optimal N treatments (Supplementary Fig. S6I). Gene expression levels were represented on heatmap by a spectrum of colours, from red (indicating the highest expression) to blue (indicating the lowest expression). There is a clear definition of the expression of these genes across the six growth stages at low N treatment in *cv*. Vikramarya and *cv.* Aditya (Fig. 7A, B).

Discussion

Since most N from applied fertilizers is lost to the environment, minimizing fertilizer use is essential for sustainable agriculture. N-efficient genotypes can be developed to improve crop plants or genotypes that can absorb and retain significant amounts of N and grow and yield well in low N regimes [39]. Such genetic modifications require a deeper knowledge of plant stress response conditions at the molecular level. The development of N-efficient genotypes necessitates a thorough knowledge of the regulatory genes

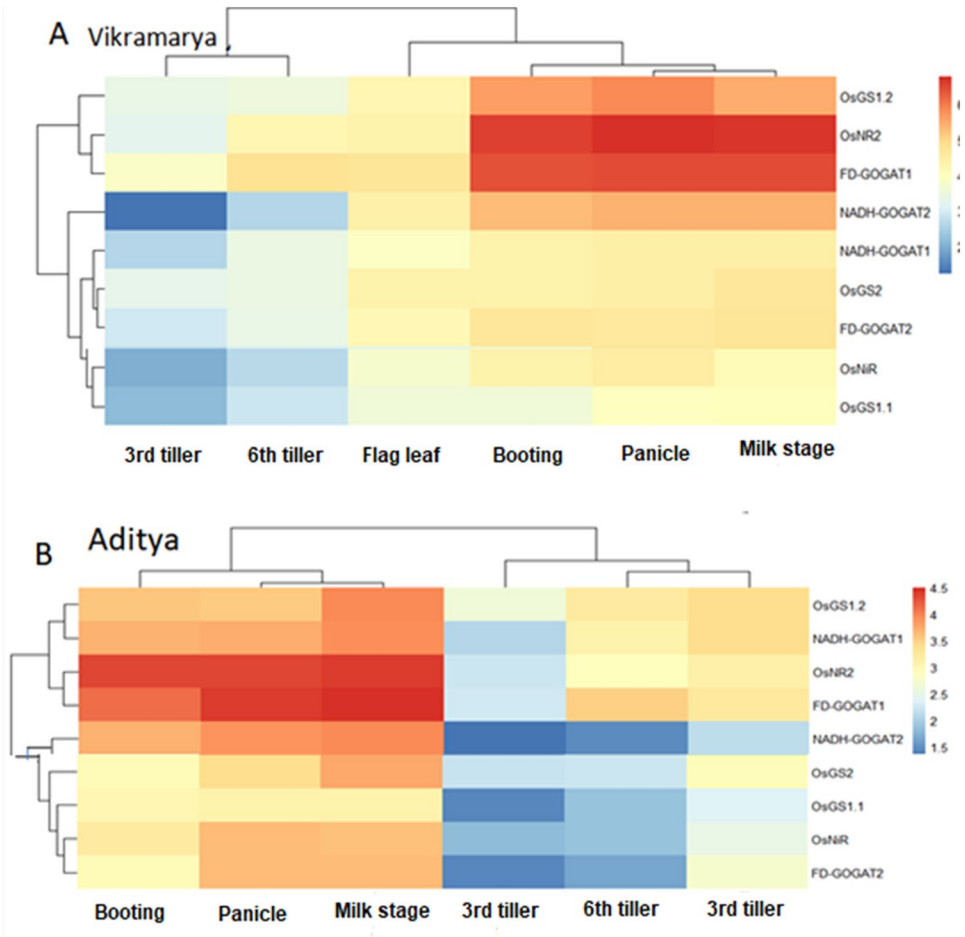

Fig. 7 Heatmap for N assimilation genes representing relative gene expression levels at different stages of growth (3rd tiller to milk stages) in cultivar Vikramarya (**A**) and Aditya (**B**) under low nitrogen treatment. The relative expression level were represented in different colour codes as given in colour key: Red (high expression) and blue (low expression). The expression levels were normalized to corresponding housekeeping gene β-actin. (Color figure online)

that impart low-N tolerance in crop plants. In the present work, screening of ten rice cultivars under low N application showed physiological differences (Supplementary Figs. S2, S3 and Tables S3, S4). The two contrasting cultivars were selected for the stage-specific experiment based on growth and physiological performances (Figs. 3 and 5; Tables 1 and 2). Phenotypic variations were observed at different growth stages in *cv*.Vikramarya and *cv.* Aditya under low N treatment. In *cv*. Aditya a significant decrease in photosynthetic efficiency, stomatal conductance, and relative chlorophyll content at higher growth stages were observed (Fig. 3), which may be due to the reduced photochemical efficiency of PSII with limiting N availability for processing of photosystems and chlorophyll molecules. The severe impact of low-N supply was observed on Fv/Fm, ΦPSII, and ETR in *cv.* Aditya than *cv.* Vikramarya; and quenching efficiency (NPQ) data suggested that *cv*. Aditya loses more light photons into heat energy due to higher values of NPQ, notably at reproductive growth stages (Table 1). However, a decrease in photosynthesis was reported at a single growth stage under low-N treatment in earlier studies [13, 40–42]. We confirmed it at different vegetative and reproductive growth stages in rice plants. Proper N-fertilization cannot alone improve the photosynthesis rate [43]. However, the maintenance of N-dependent photosynthetic components is necessary during the growth of rice plants [44]. The photochemical efficiency attributes are essential indicators for plant growth, physiological response, and modulation of PSII under low N conditions. The malfunction of PSII due to low-N supply was studied, where increased NPQ explained the dissipation of exciting energy of PSII as heat [45] and the slow rate of the PSII reaction center from quenched to unquenched state [46]. Furthermore, a significant decrease in ETR was observed in *cv.* Aditya as compared to the *cv.* Vikramarya, which may be due to low proton motive force in non-cyclic electron transport mode during the photochemical reaction [47] and, thus, transiently obstruct the photosynthesis in *cv.* Aditya, particularly at reproductive growth stages [48]. The observed results showed that reduction in photosynthetic and fluorescence efficiency impacts the growth attributes, including plant biomass, leaf area, and plant length showing more decline in *cv*. Aditya than *cv.* Vikramarya under low N treatment (Fig. 5). The Pearson's correlation matrix of physio-biochemical, chlorophyll fluorescence, and growth traits showeda significant positive correlation in both rice cultivars, but negative correlations

were also found to be more prominent in *cv.* Aditya than Vikramarya cultivar under low-N application (Supplementary Fig. S7). The leaf nitrogen content showed a strong and positive correlation with growth traits and photosynthesis, at different growth stages. Murata [49] also observed a high positive association between leaf photosynthesis and crop growth rates. Increased LNC per unit boosts rice photosynthesis and biomass production, possibly due to increased N supply to Rubisco. However, increased photosynthesis in rice plants would increase yield and biomass [13].

The observed improvements in agronomic attributes for *cv*. Vikramarya suggests that this cultivar is more successful in taking up and using N for grain growth than *cv.* Aditya. The grain yield per unit area was severely declined in *cv.* Aditya compared to *cv.* Vikramarya under low-N supply (Fig. S6), due to the low production of panicles per unit area and grains per panicle (Fig. 6A–D) in *cv.* Aditya. The leaf N levels in *cv.* Vikramarya increased by up to ~ 18% at higher growth stages when compared with the *cv.* Aditya at low-N supply (Table 2). The overall result was an enhanced NUE in *cv*. Vikramarya (Fig. 6I) due to increased NUtE and NUpE for low-N supply than *cv*. Aditya (Fig. 6G, H). These outcomes suggest that the influence of NUpE and NUtE on NUE strongly differs dependent on the amount of N fertilization and rice cultivar [24]. While other studies in cereals have also demonstrated that the NUpE influences NUE at low-N supply [50, 51], and NUtE was more closely associated with genetic variation in NUE under different N regimes rather than NUpE [13, 52, 53]. Indeed, in the current study, growth and yield enhancements in *cv.* Vikramarya versus *cv*. Aditya were detected under the low-N supply, and NUE was improved due to NUpE and NUtE in *cv*. Vikramarya than in *cv*. Aditya. Together, this supports that the high-efficient *cv.* Vikramarya and low-efficient *cv*. Aditya represent exceptional candidates for studying genetic differences pertaining to rice yield and NUE.

The enhancement in N application can promote the activities of NR and GS, the ability of N absorption and assimilation after flowering, and the content of grain protein [54]. The enzyme activities related to leaf N metabolism are directly affected by the level of soil fertilizer supply [55]. A suitable amount of nitrogen fertilizer can enrich the activities of NR, NiR, GS, and GOGAT in the leaves of rice at the later stages of growth, but higher application of nitrogen fertilizer will reduce their activities [56]. *cv.* Aditya and Vikramarya showed a drop in NR activity by 45% and 37% at reproductive phases, respectively, but an increase in activity was detected during the vegetative stage at low N treatment (Table 2). The differences in NR activity persist in contrasting cultivars at the transcript level. Leaf NR activity was higher than root activity in several cereals and other crop plants [57]. Root cell nitrate and amino acid concentrations could be influenced by the NR, plays a key role in absorption of nitrate [58]. NR activity may differ between the two cultivars because of differences in the regulation of N transporter genes or N flux in roots [59]. A decrease in NiR activity was observed during reproductive stages in both cultivars but the decline was more prominent in *cv*. Aditya (21%) than *cv.* Vikramarya (19.70%) under low N supply.

In N metabolism, GS serves as a multifunctional enzyme. In higher plants, all N goes through the GS reaction and a single nitrogen atom is subjected the GS reaction many times [60]. Assimilation and remobilization in diverse organs to the ultimate storage protein during uptake from the soil. Therefore, the GS reaction serves as a control point for N assimilation since the GS product is given to glutamate synthase in a regular amount [61]. The GS and glutamate synthase work together to direct N flow, which is then utilized by the rest of the metabolism. The GS plays an essential role in N nutrition and enhancing grain yield in rice [62]. In this study, GS activity was reduced at low N treatment and effects were more pronounced in the *cv*. Aditya (27%) than *cv.* Vikramarya, at low N application, indicating that GS plays a vital role in N uptake and utilization under low N conditions. Increase in GS activity and N content in plants grown hydroponically under low and optimum N treatment may be attributed to over-expression of GS1 isoform [63]. At low N treatments, cultivars with differential GOGAT activity showed a considerable impact. Both cultivars had significant levels of leaf GOGAT activity at the vegetative stage, but this activity decreased when the plants entered the reproductive phase. Under low N conditions, GS and NR play a key role in assimilating nutrients for crop growth [64]. The metabolic rates are determined by the enzymes activity that are involved in N assimilation [59]. The strength of GS, GOGAT, and GDH enzyme activities showed the power of plant to assimilate organic N into amino acids. Their activities are affected by different N application doses [59]. High N efficient cultivar (*cv.* Vikramarya) had greater activities in growth stages as compared to low N inefficient cultivar (*cv*. Aditya), indicating these enzymes are closely associated with N metabolism in plants. Further, a well multiplex system of N uptake and assimilation may be base for better N use efficiency in *cv.* Vikramarya owing to their high enzymatic activity (Table 2).

Genes such as NR, NiR, GS, and GOGAT are widely known to be regulated by N, which serves as a signaling source [65]. The expression level of genes related to N metabolism between an N-efficient rice *cv.* Vikramarya and a low efficient rice *cv*. Aditya under low-N treatment were analyzed and found that NR, NIR, GS, and GOGAT were highly expressed in *cv.* Vikramarya than *cv.* Aditya (Supplementary Fig. 6SA-I). These results showed that NiR, GS2, and GOGAT might play important roles in low-N tolerance in Vikramarya, especially GS2 and because of their induction in leaves of *cv.* Vikramarya at different growth stages and their stronger responses to low-N stress

in the leaves of Vikramarya than in *cv.* Aditya, which might also be an important reason for *cv.*Vikramarya better adaptation to low-N stress. Nevertheless, in barley (*Hordeum vulgare* L.), increased expression of the cytosolic , which plays a role in N remobilization, resulted in higher grain yield and NUE compared with wild-type plants when grown under varying N supply [66]. Low and high NUE cultivars differed in the expression of NiR and GS, suggesting that these enzymes play an active role in the efficient uptake and use of N fertilizer [64]. Experiments on a rice hybrid that received five N treatments showed that ammonium assimilation enzymes have an important influence on grain yield and NUE [67]. In rice seedlings, Hirose et al. [68] found that activating NADH-GOGAT expression using NH_4Cl in root tissues boosted its expression. After ammonium induction, Sonoda et al. [69] found comparable effects, with an increase in NADH-GOGAT mRNA accumulation (after 60 min). However, cytosolic GS1 expression remained stable throughout the ammonium induction process [70]. GS and GDH were shown to be expressed in barley seeds at an early stage of seed development in another investigation [71]. In order to compensate for the low expression of GS genes in seeds, Grabowska and Kwintaj [72] found that TsGS1-3 and TsGS2-1 (low), and TsGDH1 (high) were differentially expressed. These corresponding genes of nitrogen acquisition, transport, and assimilation contribute to efficient use of plant N [27]. Our results showed that rice plants from the high-efficient *cv.* Vikramarya expressed these genes more than low-efficient *cv.* Aditya at various phases of growth, explaining their role in N assimilation in rice.

Conclusion

In conclusion, two contrasting rice cultivars showed significant physiological, biochemical, and genetic variations in leaf photosynthesis, photochemical efficiency of PSII, plant growth attributes at different growth stages, grain yields, and nitrogen use efficiency (NUE) under low-N supply. The outcomes of the study support that changes in low-efficient plants are required to attain higher physiological growth and crop yields along with improved NUpE, NUtE, and NUE. These modifications are controlled at the gene level, particularly by important regulators like C and N at low N conditions. Our study further suggests that the genetic manipulation of N metabolism genes provides a potential strategy to improve NUE at low N supply. However, while the changed expression of single metabolic and transporter genes has been successful in some plant species but individual gene changes may not be suitable for a general approach to improve NUE; instead multiple gene targets approach enhances the NUE in rice [73]. Specific modulation of leaf N/C metabolic and transport processes may require efficient coordination to avoid end-product inhibition or substrate limitation of metabolic pathways and to enhance NUE.

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Declarations

Competing Interests The authors have no relevant financial or non-financial interests to disclose.

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References

1. Pandey S, Byerlee D, Dawe D, Dobermann A, Mohanty S, Rozelle S (2010) Rice in the Global Economy. Strategic Research and Policy Issues for Food Security. IRRI, Los Baños
2. Nadeem F, Mahmood R, Sabir M, Haider MS, Wang R, Zhong Y, Ishfaq M, Li X (2022) Foxtail millet [Setariaitalica (L.) Beauv.] over-accumulates ammonium under low nitrogen supply. Plant Physiol Biochem. https://doi.org/10.1016/j.plaphy.2022.05.031
3. Zhu S, Vivanco JM, Manter DK (2016) Nitrogen fertilizer rate affects root exudation, the rhizosphere microbiome and nitrogen-use-efficiency of maize. Appl Soil Ecol 107:324–333. https://doi.org/10.1016/j.apsoil.2016.07.009
4. Anas M, Liao F, Verma KK, Sarwar MA, Mahmood A, Chen ZL, Li Q, Zeng XP, Liu Y, Li Y (2020) Fate of nitrogen in agriculture and environment: agronomic, eco-physiological and molecular approaches to improve nitrogen use efficiency. Biol Res 53(1):1–20. https://doi.org/10.1186/s40659-020-00312-4
5. Mu X, Chen Q, Chen F, Yuan L, Mi G (2016) Within-leaf nitrogen allocation in adaptation to low nitrogen supply in maize during grain-filling stage. Front Plant Sci 7:699. https://doi.org/10.3389/fpls.2016.00699
6. Cen H, Weng H, Yao J, He M, Lv J, Hua S, Li H, He Y (2017) Chlorophyll fluorescence imaging uncovers photosynthetic fingerprint of citrus Huanglongbing. Front plant Sci 8:1509. https://doi.org/10.3389/fpls.2017.01509
7. Maxwell K, Johnson GN (2000) Chlorophyll fluorescence—a practical guide. J Exp Bot 51(345):659–668. https://doi.org/10.1093/jexbot/51.345.659
8. Tantray AY, Bashir SS, Ahmad A (2020) Low nitrogen stress regulates chlorophyll fluorescence in coordination with photosynthesis and Rubisco efficiency of rice. Physiol Mol Biol Plants 26(1):83–94. https://doi.org/10.1007/s12298-019-00721-0
9. Xue YU, Jia xin HU, Yu-bo WA, Zhi-wei DI, Cai-Feng LI, Zhi-jia GA, Feng-Ming MA (2014) Effect of nitrogen on chlorophyll

fluorescence of blade of sugar beet. J Nucl Agric Sci 28(10):1918. https://doi.org/10.11869/j.issn.100-8551.2014.10.1918
10. Bassi D, Menossi M, Mattiello L (2018) Nitrogen supply influences photosynthesis establishment along the sugarcane leaf. Sci Rep 8:2327. https://doi.org/10.1038/s41598-018-20653-1
11. Evans JR, Clarke VC (2018) The nitrogen cost of photosynthesis. J Exp Bot 70:7–15. https://doi.org/10.1093/jxb/ery366
12 Tendler A, Wolf BC, Tiwari V, Alon U, Danon A (2018) Fold-change response of photosynthesis to step increases of light level. iScience 8:126–137. https://doi.org/10.1016/j.isci.2018.09.019
13 Makino A (2011) Photosynthesis, grain yield, and nitrogen utilization in rice and wheat. Plant Physiol 155(1):125–129. https://doi.org/10.1104/pp.110.165076
14. Gu J, Zhou Z, Li Z, Kong X, Wang Z, Yang J (2016) Effects of the mutant with low chlorophyll content on photosynthesis and yield in rice. Acta Agron Sin 42(4):551–560. https://doi.org/10.3724/SP.J.1006.2016.00551
15. Meng X, Wang X, Zhang Z, Xiong S, Wei Y, Guo J, Zhang J, Wang L, Ma X, Tegeder M (2021) Transcriptomic, proteomic, and physiological studies reveal key players in wheat nitrogen use efficiency under both high and low nitrogen supply. J Exp Bot 72(12):4435–4456. https://doi.org/10.1093/jxb/erab153
16. Xu G, Fan X, Miller AJ (2012) Plant nitrogen assimilation and use efficiency. Annu Rev Plant Biol 63:153–182. https://doi.org/10.1146/annurev-arplant-042811-105532
17. Griffiths CA, Paul MJ, Foyer CH (2016) Metabolite transport and associated sugar signalling systems underpinning source/sink interactions. Biochem Biophys Acta 1857(10):1715–1725. https://doi.org/10.1016/j.bbabio.2016.07.007
18 Martin T, Oswald O, Graham IA (2002) Arabidopsis seedling growth, storage lipid mobilization, and photosynthetic gene expression are regulated by carbon: nitrogen availability. Plant Physiol 128(2):472–481. https://doi.org/10.1104/pp.010475
19. Melo-Oliveira R, Oliveira IC, Coruzzi GM (1996) Arabidopsis mutant analysis and gene regulation define a nonredundant role for glutamate dehydrogenase in nitrogen assimilation. Proc Natl Acad Sci USA 93(10):4718–4723. https://doi.org/10.1073/pnas.93.10.471
20. Moll RH, Kamprath EJ, Jackson WA (1982) Analysis and interpretation of factors which contribute to efficiency of nitrogen utilization. Agron J 74:562–564. https://doi.org/10.2134/agronj1982.00021962007400030037x
21. Tegeder M, Masclaux-Daubresse C (2018) Source and sink mechanisms of nitrogen transport and use. New Phytol 217:35–53. https://doi.org/10.1111/nph.14876
22. Dechorgnat J, Nguyen CT, Armengaud P, Jossier M, Diatloff E, Filleur S, Daniel-Vedele F (2011) From the soil to the seeds: the long journey of nitrate in plants. J Exp Bot 62:1349–1359. https://doi.org/10.1093/jxb/erq409
23. Masclaux-Daubresse C, Chardon F (2011) Exploring nitrogen remobilization for seed filling using natural variation in *Arabidopsis thaliana*. J Exp Bot 62:2131–2142. https://doi.org/10.1093/jxb/erq405
24. Idowu O, Wang Y, Homma K, Nakazaki T, Xu Z, Shiraiwa T (2022) Interaction of erect panicle genotype and nitrogen fertilizer application on the source-sink ratio and nitrogen use efficiency in rice. Field Crops Res 278:108430. https://doi.org/10.1016/j.fcr.2022.108430
25 Barneix AJ (2007) Physiology and biochemistry of source-regulated protein accumulation in the wheat grain. J Plant Physiol 164(5):581–590. https://doi.org/10.1016/j.jplph.2006.03.009
26. Wang H, McCaig TN, DePauw RM, Clarke JM (2008) Flag leaf physiological traits in two high-yielding Canada Western Red Spring wheat cultivars. Can J Plant Sci 88:35–42. https://doi.org/10.4141/CJPS07055
27 Krapp A (2015) Plant nitrogen assimilation and its regulation: a complex puzzle with missing pieces. Curropin Plant Biol 25:115–122. https://doi.org/10.1016/j.pbi.2015.05.010
28. Gaufichon L, Masclaux-Daubresse CÉ, Tcherkez G, Reisdorf-Cren MI, Sakakibara Y, Hase T, Clement G, Avice JC, Grandjean O, Marmagne A, Boutet-Mercey ST (2013) *Arabidopsis thaliana* ASN2 encoding asparagine synthetase is involved in the control of nitrogen assimilation and export during vegetative growth. Plant Cell Environ 36(2):328–342. https://doi.org/10.1111/j.1365-3040.2012.02576.x
29. Sinha SK, Chaudhary S, Tyagi P, Venkadesan S, Rani M, Mandal PK (2018) Transcriptome analysis of two rice varieties contrasting for nitrogen use efficiency under chronic N starvation reveals differences in chloroplast and starch metabolism-related genes. Genes 9(4):206. https://doi.org/10.3390/genes9040206
30. Li Q, Ding G, Yang N, White PJ, Ye X, Cai H, Lu J, Shi L, Xu F (2020) Comparative genome and transcriptome analysis unravels key factors of nitrogen use efficiency in *Brassica napus* L. Plant Cell Environ 43(3):712–731. https://doi.org/10.1111/pce.13689
31. Bacilio M, Moreno M, Lopez-Aguilar DR, Bashan Y (2017) Scaling from the growth chamber to the greenhouse to the field: demonstration of diminishing effects of mitigation of salinity in peppers inoculated with plant growth-promoting bacterium and humic acids. Appl Soil Ecol 119:327–338. https://doi.org/10.1016/j.apsoil.2017.07.002
32. Bennett E, Roberts JA, Wagstaff C (2012) Manipulating resource allocation in plants. J Exp Bot 63(9):3391–3400. https://doi.org/10.1093/jxb/err442
33. Kong L, Xie Y, Hu L, Feng B, Li S (2016) Remobilization of vegetative nitrogen to developing grain in wheat (Triticum aestivum L.). Field Crop Res 196:134–144. https://doi.org/10.1016/j.fcr.2016.06.015
34 Yoshida S (1981) Fundamentals of rice crop science. International Rice Research Institute, Los Baños
35. Mahajan GR, Pandey RN, Sahoo RN, Gupta VK, Datta SC, Kumar D (2017) Monitoring nitrogen, phosphorus and sulphur in hybrid rice (*Oryza sativa* L.) using hyperspectral remote sensing. Precis Agric 18(5):736–761. https://doi.org/10.1007/s11119-016-9485-2
36. Jin CW, Du ST, Shamsi IH, Luo BF, Lin XY (2011) NO synthase-generated NO acts downstream of auxin in regulating Fe-deficiency-induced root branching that enhances Fe-deficiency tolerance in tomato plants. J Exp Bot 62(11):3875–3884. https://doi.org/10.1093/jxb/err078
37. Sun HY, Qian Q, Wu K, Luo JJ, Wang SS, Zhang CW, Ma YF, Liu Q, Huang XZ, Yuan QB (2014) Heterotrimeric G proteins regulate nitrogen-use efficiency in rice. Nat Genet 46:652–656. https://doi.org/10.1038/ng.2958
38 Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25(4):402–408. https://doi.org/10.1006/meth.2001.1262
39. Delmer DP (2005) Agriculture in the developing world: connecting innovations in plant research to downstream applications. Proc Nat Acad Sci USA 102(44):15739–15746. https://doi.org/10.1073/pnas.050589510
40. Fuentes SI, Allen DJ, Ortiz-Lopez A, Hernández G (2001) Overexpression of cytosolic glutamine synthetase increases photosynthesis and growth at low nitrogen concentrations. J Exp Bot 52:1071–1081. https://doi.org/10.1093/jexbot/52.358.1071
41. Pan S, Liu H, Mo Z, Patterson B, Duan M, Tian H, Hu S, Tang X (2016) Effects of nitrogen and shading on root morphologies, nutrient accumulation, and photosynthetic parameters in different rice genotypes. Sci Rep 6:1–14. https://doi.org/10.1038/srep32148
42. Tantray AY, Hazzazi Y, Ahmad A (2022) Physiological, agronomical, and proteomic studies reveal crucial players in rice nitrogen use efficiency under low nitrogen supply. Int J Mol Sci 23(12):6410. https://doi.org/10.3390/ijms23126410

43. Peng S, Buresh RJ, Huang J, Zhong X, Zou Y, Yang J, Wang G, Liu Y, Hu R, Tang Q et al (2010) Improving nitrogen fertilization in rice by site specific N management. A review. Agron Sustain Dev 30:649–656. https://doi.org/10.1051/agro/2010002
44. Yang H, Ge CS, Ying W, Yang JP, Li JW, He JJ (2014) Effect of shading on leaf SPAD values and the characteristics of photosynthesis and morphology of rice canopy. PLoS ONE 9(2):e88421. https://doi.org/10.1371/journal.pone.0088421
45. Müller P, Li XP, Niyogi KK (2001) Non-photochemical quenching. A response to excess light energy. Plant Physiol 125:1558–1566. https://doi.org/10.1104/pp.125.4.1558
46. Kromdijk J, Głowacka K, Leonelli L, Gabilly ST, Iwai M, Niyogi KK, Long SP (2016) Improving photosynthesis and crop productivity by accelerating recovery from photoprotection. Science 354:857–861. https://doi.org/10.1126/science.aai8878
47. Ferreira PA, Ceretta CA, Soriani HH, Tiecher TL, Soares CR, Rossato LV, Nicoloso FT, Brunetto G, Paranhos JT, Cornejo P (2015) Rhizophagus clarus and phosphate alter the physiological responses of *Crotalaria juncea* cultivated in soil with a high Cu level. Appl Soil Ecol 91:37–47. https://doi.org/10.1590/18069657rbcs20170245
48. Govindjee, U (2014) Non-photochemical quenching and energy dissipation in plants, algae and cyanobacteria. In: Demmig-Adams B, Garab G, Adams W III (eds). Springer, Dordrecht, vol 40, pp 297–314. https://doi.org/10.1007/978-94-017-9032-1
49 Murata Y (1981) Dependence of potential productivity and efficiency for solar energy utilization on leaf photosynthetic capacity in crop species. Jpn J Crop Sci 50(2):223–232. https://doi.org/10.1626/jcs.50.223
50. Muurinen S, Slafer GA, Peltonen-Sainio P (2006) Breeding effects on nitrogen use efficiency of spring cereals under northern conditions. Crop Sci 46:561–568. https://doi.org/10.2135/cropsci2005-05-0046
51. Liang Z, Bronson KF, Thorp KR, Mon J, Badaruddin M, Wang G (2014) Cultivar and N fertilizer rate affect yield and N use efficiency in irrigated durum wheat. Crop Sci 54:1175–1183. https://doi.org/10.2135/cropsci2013.03.0159
52. Barraclough PB, Lopez-Bellido R, Hawkesford MJ (2014) Genotypic variation in the uptake, partitioning and remobilisation of nitrogen during grain-filling in wheat. Field Crops Res 156:242–248. https://doi.org/10.1016/j.fcr.2013.10.004
53. Tegeder M, Perchlik M (2018) The importance of organic nitrogen transport processes for plant productivity and nitrogen use efficiency. In: Shrawat AK, Zayed A, Lightfoot DA (eds) Engineering nitrogen utilization in crop plants. Springer, New York, pp 233–253. https://doi.org/10.1007/978-3-319-92958-3_13
54. Wang YF, Yu ZW, Li SX, Yu SL (2002) Effect of nitrogen nutrition level on the activity of key enzymes of nitrogen metabolism and the content of egg self quality in winter wheat. Acta Agron Sin 28(6):743–748
55 Tischner R (2000) Nitrate uptake and reduction in higher and lower plants. Plant Cell Environ 23(10):1015–1024. https://doi.org/10.1046/j.1365-3040.2000.00595.x
56. Li W, Lv Y, Liu X, Tong T, Cao X, Gu W, Wei S (2018) Effects of nitrogen fertilizer on nitrogen metabolizing enzymes, nitrogen utilization and yield of maize with different nitrogen efficiency. Southwest China J Agric Sci 31:1829–1835
57. Oaks A (1979) Nitrate assimilation in roots and its regulation. In: Hewitt EJ, Culling CV (eds) Nitrogen assimilation of plants. Academic Press, London, pp 217–226
58. Hakeem KR, Ahmad A, Iqbal M, Gucel S, Ozturk M (2011) Nitrogen-efficient rice cultivars can reduce nitrate pollution. Environ Sci Pollut Res 18(7):1184–1193. https://doi.org/10.1007/s11356-010-0434-8
59. Shah JM, Bukhari SA, Zeng JB, Quan XY, Ali E, Muhammad N, Zhang GP (2017) Nitrogen (N) metabolism related enzyme activities, cell ultrastructure and nutrient contents as affected by N level and barley genotype. J Int Agric 16(1):190–198. https://doi.org/10.1016/S2095-3119(15)61308-9
60. Coque M, Gallais A (2006) Genomic regions involved in response to grain yield selection at high and low nitrogen fertilization in maize. Theoret Appl Genet 112(7):1205–1220. https://doi.org/10.1007/s00122-006-0222-5
61. Mokhele B, Zhan X, Yang G, Zhang X (2012) Nitrogen assimilation in crop plants and its affecting factors. Can J Plant Sci 92(3):399–405. https://doi.org/10.4141/cjps2011-135
62. Jin ZX, Qian CR, Jing Y, Liu HY, Piao ZZ (2007) Changes in activities of glutamine synthetase during grain filling and their relation to rice quality. Rice Sci 14(3):211–216. https://doi.org/10.1016/S1672-6308(07)60029-8
63. Cai H, Zhou Y, Xiao J, Li X, Zhang Q, Lian X (2009) Overexpressed glutamine synthetase gene modifies nitrogen metabolism and abiotic stress responses in rice. Plant Cell Rep 28(3):527–537. https://doi.org/10.1007/s00299-008-0665-z
64. Hakeem KR, Chandna R, Ahmad A, Qureshi M, Iqbal M (2012) Proteomic analysis for low and high nitrogen-responsive proteins in the leaves of rice genotypes grown at three nitrogen levels. Appl Biochem Biotechnol 168(4):834–850. https://doi.org/10.1007/s12010-012-9823-4
65. Crawford NM (1995) Nitrate: nutrient and signal for plant growth. Plant Cell 7(7):859–868. https://doi.org/10.1105/tpc.7.7.859
66. Gao Y, de Bang TC, Schjoerring JK (2019) Cisgenic overexpression of cytosolic glutamine synthetase improves nitrogen utilization efficiency in barley and prevents grain protein decline under elevated CO_2. Plant Biotechnol J 17:1209–1221. https://doi.org/10.1111/pbi.13046
67. Sun Y, Ma J, Sun Y, Xu H, Yang Z, Liu S, Jia X, Zheng H (2012) The effects of different water and nitrogen managements on yield and nitrogen use efficiency in hybrid rice of China. Field Crops Res 127:85–98. https://doi.org/10.1016/j.fcr.2011.11.015
68. Hirose N, Hayakawa T, Yamaya T (1997) Inducible accumulation of mRNA for NADH-dependent glutamate synthase in rice roots in response to ammonium ions. Plant Cell Physiol 38(11):1295–1297. https://doi.org/10.1093/oxfordjournals.pcp.a029120
69. Sonoda Y, Ikeda A, Saiki S, Wirén NV, Yamaya T, Yamaguchi J (2003) Distinct expression and function of three ammonium transporter genes (OsAMT1, 1–1, 3) in rice. Plant Cell Physiol 44(7):726–734. https://doi.org/10.1093/pcp/pcg083
70 Tobin AK, Yamaya T (2001) Cellular compartmentation of ammonium assimilation in rice and barley. J Exp Bot 52(356):591–604. https://doi.org/10.1093/jexbot/52.356.591
71. Hansen TH, Laursen KH, Persson DP, Pedas P, Husted S, Schjoerring JK (2009) Micro-scaled high-throughput digestion of plant tissue samples for multi-elemental analysis. Plant Methods 5(1):1–11. https://doi.org/10.1186/1746-4811-5-12
72. Grabowska A, KwintaJ BW (2012) Glutamine synthetase and glutamate dehydrogenase in triticale seeds: molecular cloning and genes expression. Acta Physiol Plant 34(6):2393–2406. https://doi.org/10.1007/s11738-012-1085-9
73. The SV, Snyder R, Tegeder M (2021) Targeting nitrogen metabolism and transport processes to improve plant nitrogen use efficiency. Front Plant Sci 11:628366. https://doi.org/10.3389/fpls.2020.628366