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Research paper

Enhanced expression of *OsSPL14* gene and its association with yield components in rice (*Oryza sativa*) under low nitrogen conditions

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ABSTRACT

Nitrogen use efficiency (NUE) in rice crop is the need of the hour for reduction of nitrous oxide emission resulting from excess nitrogen (N) fertilizer application and also in reduction of cost of cultivation. Ten rice genotypes were grown under low and recommended dose of N application and characterized in terms of parameters related to yield, yield related components and NUE indicators. Wide genetic variability under low N conditions was observed with significant variation for 15 yield related parameters in interactions of genotypes and treatment. Limitation of N has led to the decrease of all yield and yield related parameters, but for grain filling % and 1000 grain weight. Two genotypes, Rasi and Varadhan have shown minimum differences between low and recommended N conditions. Correlation analysis of various yield components showed the importance of the secondary branches for the total grains under low N. Expression analysis of *OsSPL14* (LOC_Os08g39890) gene reported to be associated with increased panicle branching and higher grain yield through real time PCR in leaf and three stages of panicle has shown differential temporal expression and its association with yield and yield related components across the genotypes. The expression of *OsSPL14* at panicle stage 3, has shown correlation ($P < 0.05$) with N% in grain. Since *OsSPL14* is a functional transcription activator, its association of expression in leaf and three panicle stages with yield components as observed in the present study suggests the role of nitrogen metabolism related genes in plant growth and development and its conversion into yield components in rice.

1. Introduction

Rice is the one of the major staple food crops across the world with a cultivated area of ~164 million ha and a production of ~740 million tonnes (www.fao.org). India has the world's largest area under rice with ~44 million ha and is the second largest producer (~105 million tonnes during 2013–14) after China. Within the country, rice occupies one-quarter of the total cropped area, contributes about 40 to 43% of total food grain production and continues to play a key role in the national food and livelihood security system. At the current rate of population growth of ~2%, population of India is expected to touch 1.63 billion by 2050 with per capita demand of ~250 g/day for rice; thus the country would require ~150 million tonnes of rice. Therefore, rice productivity needs to be enhanced from the present 2.05 t ha^{-1} to 3.3–4.05 t ha^{-1} in the next 40 years to keep pace with the increasing demand for rice and constraints for rice production (DRR Vision 2050). The increase of the productivity has been simultaneous with increase of inputs especially fertilizers.

Among the major fertilizer inputs, nitrogen (N) is the key nutrient element required in large quantities by rice. Nitrogen is a primary constituent of nucleotides, amino acids, proteins, chlorophyll and several plant hormones and is a crucial macronutrient essential and rate-limiting for the growth and development of plants. India is the third largest producer and second largest consumer of chemical fertilizers in the world. Fertilizer consumption increased from 70 thousand tonnes in 1950–51 to more than 28 million tonnes by 2012 and more than 65% of it is N fertilizers. Out of the total amount of applied N, only 30–40% of the applied N reaches the plant (Good et al., 2004; Hakeem et al., 2011; Prasad, 2013; Raun and Johnson, 1999) and the remaining is lost to the environment. In irrigated lowland rice, N losses are rapid because of ammonia volatization, denitrification, surface runoff and leaching in soil-floodwater system (De Datta and Buresh, 1989, Prasad, 2013). Leaching of nitrogen to water table causes water contamination and eutrophication of water bodies (London, 2005; Hirel et al., 2007). Through fertilizer management strategies, considerable progress has been achieved to reduce N losses by new application methods and modified N sources in the past decades. However, increase in global rice yield was not kept in pace with the fertilizer consumption thus increasing the production costs of rice. In addition, the excess nitrogen fertilizer use increases environmental pollution and the cost of the rice

Abbreviations: N, nitrogen; *OsSPL14*, *Squamosa promoter binding protein like-14*; NUE, nitrogen use efficiency; AE, Agronomic Efficiency; APE, Agro-Physiological Efficiency; UE, Utilization Efficiency; ARE, Apparent Recovery Efficiency; ANOVA, analysis of variance; LSD, least significant difference.

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cultivation. Recent awareness about the climate change and the role of greenhouse gases viz., methane and nitrous oxide emitting from rice fields has emphasized the rational use of nitrogen fertilization. Nitrous oxide is 310 times more potent greenhouse gas than CO_2 and 21 times potent than methane on a 100 year time scale, though atmospheric loading of nitrous dioxide is low (www.ipcc.ch). In India, the current average nitrogen use efficiency (NUE) in the field is approximately 33% and a substantial proportion of the remaining 67% is lost into the environment, especially in the intensively cropped areas (Abrol et al., 2007).

With the priority of the low input sustainable rice cultivation for environment friendly agriculture, NUE of rice becomes the need of the hour. Though efficient fertilizer management practices may improve nutrient use efficiency, unless the cultivar is responsive, there is a limited scope of adopting those costly or labor intensive practices by the farmers. Thus, developing rice genotypes with high NUE becomes one of the major objectives of rice breeding programs in current agricultural scenario. High NUE cultivars can be defined by their ability to produce higher grain yields under low N inputs (Ladha et al., 1998). Thus, the idea is to get the maximum possible yield with optimum inputs of nitrogen compensating the compromised yield with economic and environmental benefits.

The candidate genes of N metabolism in rice are well characterized and differential expression of several candidate genes directly involved in N metabolism and transcription factors in relation to NUE has been earlier reported in rice under differential N applications (Cao et al., 2008; Duan et al., 2007; Fan et al., 2007; Hakeem et al., 2012; Kumar et al., 2003; Li et al., 2006; Shi et al., 2010; Qiu et al., 2009; Zhao et al., 2012; Zhao and Shi 2006).

With the advent of various gene identification and positional cloning strategies, several genes involved in yield have been identified (Ikeda et al., 2013). Of these, *OsSPL14* gene (Squamosa promoter binding protein-like 14) (LOC_Os08g39890; RAP ID Os08g0509600) found to be associated with ideal plant architecture, panicle branching, higher grain productivity and reduction in tiller number. Analysis of *OsSPL14* gene showed a target site for a micro-RNA viz., OsmiRNA156 and mutation/s are also reported in the target site of OsmiRNA156. In some rice cultivars, OsmiRNA156 binds to *OsSPL14* transcripts and cleaves them, however, if mutation/s are there in the target site of OsmiRNA156 of *OsSPL14*, transcripts are accumulated leading to the increased number of spikelets (Jiao et al., 2010; Luo et al., 2012; Miura et al., 2010).

Studies showed that N plays a critical role during panicle initiation, spikelet differentiation and heading time by increasing the number of differentiated spikelets, preventing differentiated spikelets from degeneration and increasing the percentage of filled grains (Mae, 1997; Matsushima, 1980). Most of the reported yield component genes also are associated with number of panicles, panicle branching pattern, spikelet number, grain number, grain size and grain filling (Ikeda et al., 2013). Thus, with the involvement of nitrogen in the enhancement of yield components and the available information on cloned yield genes, an attempt was made to study the expression of *OsSPL14* gene in leaf and three stages of panicle of 10 genotypes grown under low N and recommended N conditions.

2. Materials and methods

2.1. Material

For the experimental material, eight released varieties/hybrids viz., Aditya (100 days), KRH2 (135 days), Krishnahamsa (125 days), Rasi (115 days), RP Bio 226 (150 days), Tellahamsa (115 days), Tulasi (100 days), Varadhan (125 days), and two advanced breeding lines viz., RP Bio 4919–377-13 (135 days) and RP Bio 4919–458 (135 days) were grown under zero and recommended dose of nitrogen application during dry season (Rabi) 2011–2012 at the experimental fields of the Indian Institute of Rice Research, Hyderabad, India. The experimental soil characteristics were slightly alkaline (pH 8.2), non-saline [electrical conductivity (EC) 0.7 dS m^{-1}], calcareous [free calcium carbonate (CaCO3) 5.01%], with cation exchange capacity (CEC) 44.1 Cmol kg^{-1} soil and medium soil organic carbon (0.69%) content. Soil has low available nitrogen (N) (102 mg kg^{-1}); high available phosphorus (P) (20.5 mg kg^{-1}) and high available potassium (K) (197 mg kg^{-1}).

2.2. Sampling and measurements

The experiment was conducted with a split plot design, without N application and with N application as main plots and genotypes as subplots in three replications. Nitrogen fertilizer @ 100 kg ha^{-1} was supplied in the form of urea (46.5%) in three equal split applications to the recommended N treatment (at basal, maximum tillering and panicle initiation stages). Phosphorus (@40 kg ha^{-1}), potassium (@40 kg ha^{-1}) and zinc (@25 kg ha^{-1}) were applied to both plots. The plot size was 15 m^2. Low N condition in the plots was maintained by not applying any nitrogenous fertilizer during the current experiment and also during last three years (2008–2011). The two main plots were separated with polyethylene film by 60 cm underground, to avoid nutrient flow between the plots.

Rice seeds were sown as nursery and one month old seedlings were transplanted at a spacing of 20 × 15 cm. From each plot, six representative hills were harvested at maturity and were divided into vegetative and reproductive parts, dried and weighed for determining dry matter of various plant parts. Grain and straw yields were adjusted to 14% grain moisture content and expressed in t ha^{-1}. Straw and grain samples were analyzed for nitrogen with Kjeldhalh method. Tillers per m^2, panicles per m^2, grain yield (t ha^{-1}), productivity per day, N % in grains and straw, total number of spikelets and grains per panicle, spikelets, grains and grain filling (%) of primary and secondary branches of upper and lower portions of the panicle, 1000 grain weight (g) and straw yield (t ha^{-1}) were recorded. Agronomic Efficiency (AE), Physiological Efficiency (PE), Agro-Physiological Efficiency (APE), Apparent Recovery Efficiency (ARE) and Utilization Efficiency (UE) using grain yield and total N uptake were calculated according to Fageria and Baligar (2003).

2.3. Data analysis

Two way analysis of variance (ANOVA) was performed using an open source software R (R Core Team, 2012) with agricolae package (Felipe de Mendiburu, 2012). Correlations among all the parameters were also calculated. Statistical significance of the parameter means was determined by performing Fisher's Least Significant Difference (LSD) test.

2.4. RNA isolation and c-DNA synthesis

Samples of leaf during maximum tillering stage (stage 1) (RT-RQ-L), panicles during maximum tillering stage (stage 1) (RT-RQ-PS1), one week after stage 1 (stage 2) (RT-RQ-PS2) and one week after stage 2 (stage 3) (RT-RQ-PS3) were collected and immediately frozen in liquid nitrogen for RNA isolation. Total RNA was isolated using TRIzol reagent (Invitrogen, USA) and the quality of the RNA was assessed using Nanodrop® ND1000 spectrophotometer (Thermo Scientific, USA). RNA samples were treated with RNAse free DNAse (Invitrogen, USA). Approximately 1 μg of total RNA from each sample was used as template for the first-strand cDNA synthesis, using Superscript III reverse transcriptase (Invitrogen, USA).

2.5. Primer design and reverse transcriptase PCR

OsSPL14 (LOC_Os08g39890) gene sequence was obtained from GenBank (http://www.ncbi.nlm.nih.gov/nuccore/JN193288.1) and gene-specific primer (forward primer: CAAACCCCTTTGGCATCAC,

reverse primer: GCGGCACTGTGGGTAGTAGT) was designed using Primer 3.0 (http://simgene.com/Primer3). Reverse transcriptase PCR was performed for samples of all stages with *Actin* and *OsSPL14* using ABI-Veriti 96 well thermal cycler (Life Technologies, USA) in 10 μl reaction volume comprising 30 ng cDNA, 10 mmol of forward and reverse primer, 10× Taq buffer (20 mM Mg^{2+}), 10 mM dNTP and 0.5 U Taq polymerase (Bangalore Genei, India). The cycling conditions included initial denaturation at 94 °C for 4 min, followed by 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C for 35 cycles, followed by final extension at 72 °C for 7 min. Amplified products were resolved on 2% Metaphor agarose gel (Lonza, USA). Gels were stained in 0.5 mg/ml ethidium bromide and documented using Alpha Imager 1220 (Alpha Innotech, USA).

2.6. Real-time quantitative RT-PCR (qRT-PCR) analysis

qRT-PCR was performed using Applied Biosystems 7500 Real Time PCR (Life Technologies, USA), in a final volume of 20 μl, containing 10 μl of Platinum® SYBR® Green qPCR SuperMix (Invitrogen, USA) with 500 nM each of forward and reverse primers and 20 ng of the cDNA samples. The real time PCR cycling conditions included a pre-incubation at 50 °C for 2 min and denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. qRT-PCR was performed as three biological replicates. Samples were run in duplicates on the same plate along with controls set up for each sample in duplicate using 18 s RNA gene. The rice 18 s RNA gene (forward primer: CTACGTCCCTGCCCTT TGTAC, reverse primer: ACACTTCACCGGACCATTCAA) was used to normalize gene expressions. The data were analyzed using the 7500 Sequence Detection Software (Applied Biosystems, USA) with default baseline and threshold. The relative expression levels of genes were calculated using the $2^{-\Delta CT\Delta CT}$ method, which represents the difference of CT between the control products and the target gene products. Recommended N situation was taken as control and low N was considered as treated.

2.7. Sequencing of Osmir156 motif region

Genomic primers were designed for *OsSPL14* gene (LOC_ Os08g39890) (*forw*ard primer: CCTCTACAGAGACCAATCCA, reverse primer: TAGCTCCTCATGGTCACTCT) spanning miR156 target motif. PCR was performed for all the 10 genotypes using ABI-Veriti 96 well thermal cycler (Life Technologies, USA) in 20 μl reaction volume comprising 30 ng DNA, 10 mmol of forward and reverse primer), 10 x Taq buffer (20 mM Mg^{2+}), 10 mM dNTPs each and 1 U Taq polymerase (Bangalore Genei, India). The cycling conditions included initial denaturation at 94 °C for 4 min, followed by 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C for 35 cycles, followed by final extension at 72 °C for 7 min. The PCR products were sequenced using ABI 3730XL DNA Analyzer (Applied Biosystems, USA) for both strands.

3. Results and discussion

3.1. Variability in yield related parameters and NUE indicators under low N

Wide variation was observed for various 21 yield and yield related parameters in 10 genotypes studied under low and recommended N with a general trend of decrease for all the yield and yield related parameters in genotypes grown under low N (Table 1) (Supplementary Table 1). ANOVA of data from low and recommended N showed significant differences among genotypes for 19 parameters (Table 1) but for grain filling % of primary branches of upper portion of the panicle and grains on secondary branches of lower portion of the panicle. The interaction between treatment and genotypes have shown significant variation for 15 yield related parameters but for total grains, grains on primary and secondary branches of lower portion of the panicle and grain filling % of spikelets on primary branches of lower portion of the panicle. A significant interaction between genotypes and nitrogen treatments was obtained for most of the parameters indicating that genotypes differed in their response to nitrogen dosage. Several earlier studies showed significant genotypic variation in terms of yield response across the nitrogen dosage treatments suggesting the existence of genotypic variability for yield response under low nitrogen (Gueye and Becker 2011; Kanfany et al., 2014; Mahajan et al., 2012; Singh et al., 1998; Tirol Padre et al., 1996). Genotypic variation for real time expression of *OsSPL14* was found to be highly significant ($P < 0.01$) across all the tissues. Only Agronomic Efficiency and Agro-Physiological Efficiency were found to significantly different among genotypes indicating the role of grain yield in NUE indicators.

The observed trend of decrease for all the yield and yield related parameters in genotypes grown under low N is expected as N content is crucial for cell/tissue expansion and multiplication, thus the limitation of N would impose constraint in total biomass and there by yield (Hirel et al., 2007). The differences between minimum mean values of low N and recommended N are always relatively less than the differences between maximum mean values of low and recommended N suggesting that the genotype will try to express its potential even under minimum inputs. However, with the uptake and utilization of N, the potential is expressed at its maximum in terms of biomass. Our observations were substantiated by earlier reports that the lines give similar yield under non-fertilized condition, but show great variability of yield under fertilized conditions (Inthapanya et al., 2000; Mahajan et al., 2010; Singh et al., 1998).

Among the genotypes, the mean difference was minimum between low and recommended N in Varadhan (duration—117 days) for panicles m^{-2}, straw N%, total spikelets, spikelets and grains on primary and secondary branches of upper portion of the panicle and total spikelets. Maximum total nitrogen content was noted in Varadhan under low N but not under recommended N suggesting the uptake efficiency of Varadhan under low N conditions. In Rasi (duration—111 days) also, the difference was minimum for grain N%, spikelets and grains on primary branches of lower portion of the panicle and total grains.

In the present study, tillers m^{-2} reduced by 27.8% under low nitrogen situation as reported in the earlier studies (Gueye and Becker 2011; Singh et al., 1998; Tirol-Padre et al., 1996). Nitrogen application described to be associated with promotion of tillering in rice with the significant different number of tillers between the basal and additional N fertilization as well as the top dressing (Lee et al., 2010; Wells and Faw, 1978). The studies have also shown that an optimum concentration of N > 35 g/kg was needed for activation of tillering and concentrations below 25 N/kg could stop tillering and further lower concentration would lead to the death of tillers (Evans, 1975).

Grain yield of rice is the final product of yield components viz., the number of panicles per unit area, the number of spikelets per panicle, the percentage of filled spikelets and the grain weight (Yoshida, 1983). Around 40% reduction of grain yield under low N conditions was observed in the present study. Increase in grain yield (t ha^{-1}) with the increase of N up to a level was earlier reported by several studies including tropical and Mediterranean regions (Artacho et al., 2009; Broadbent et al., 1987; De Datta et al., 1990; Geoffrey et al., 2012; Gueye and Becker 2011; Inthapanya et al., 2000; Kanfany et al., 2014; Koutroubas and Ntanos 2003; Mahajan et al., 2010; Mahajan et al., 2012; Mae et al., 2006; Metwally et al., 2010; Samonte et al., 2006; Singh et al., 1998; Sui et al., 2013; Tirol-Padre et al., 1996; Yoshinaga et al., 2013; Zhang et al., 2013; Zhao et al., 2012.).

Panicles m^{-2} were reduced by 22.4% under low N situation in the present study. The stimulation of tillering by nitrogen also enhances the number of panicles. An increase of 39.2% was observed for productive tillers for aromatic rice with 40 kg N ha^{-1} in comparison with zero N application (Mahajan et al., 2010). Similar results of significant increase of panicles m^{-2} with the increase of N application were also

Table 1
ANOVA of yield components, nitrogen content, real time expression of *OsSPL14* and NUE indicators in low and recommended N conditions.

	Range		Mean				
	Low N	Rec N	Low N	Rec N	Treatment (T)	Entry (E)	T × E
Tillers m^{-2}	165–382	204–561	275	381	***	***	*
Panicles m^{-2}	139–343	165–535	252.1	349.1	***	***	*
Upper primary spikelets	8–42	10–31	18.4	21.5	*	***	**
Upper primary grains	6–31	8–30	16.9	19.9	**	***	**
Upper primary GF%	73.8–100	73.6–100	93.1	91.9	ns	**	**
Upper secondary spikelets	8–39	9–62	23.2	29.7	***	***	**
Upper secondary grains	8–39	9–47	21.8	25.7	*	***	*
Upper secondary GF%	75–100	47.8–100	94.9	87.8	**	ns	*
Lower primary spikelets	9–52	12–56	26.2	31.6	***	***	*
Lower primary grains	9–39	10–46	23.9	27.5	*	***	ns
Lower primary GF%	27.6–102	68.5–100	55.7	87.7	***	ns	ns
Lower secondary spikelets	8–62	10–81	35.1	40.6	*	***	*
Lower secondary grains	8–56	10–60	30.2	32.1	ns	***	ns
Lower secondary GF%	3.9–12.3	71.9–141.8	7.5	99.9	***	***	***
Total spikelets $panicle^{-1}$	59–165	52–202	102.6	122.8	***	***	***
Total grains $panicle^{-1}$	55–139	44–165	92.6	104.1	**	***	ns
Total GF% $panicle^{-1}$	71.6–100	64.2–100	90.9	85.5	***	***	***
N % in grain	0.8–1.0	0.92–1.16	0.9	1	***	***	***
N % in straw	0.3–0.8	0.3–0.8	0.5	0.6	**	ns	ns
1000 grain weight (g)	11.9–24.3	11.99–25.8	20.3	21	*	***	ns
Straw yield (t ha^{-1})	1.5–3.8	3.6–7.25	2.7	5	***	*	ns
Grain yield (t ha^{-1})	2.4–3.8	3.63–6.15	3.1	5.1	***	***	***
Productivity per day	20.3–28.4	28.3–45.4	24.3	40.2	***	***	***
RT-RQ-Leaf			1.5			***	
RT-RQ-PS1			2.5			***	
RT-RQ-PS2			1			***	
RT-RQ-PS3			1.6			***	
AE (kg kg^{-1})			20.5			***	
PE (kg kg^{-1})			106.7			ns	
APE (kg kg^{-1})			51			*	
ARE (%)			41.6			ns	
UE (kg kg^{-1})			43.4			ns	

Data indicate mean of 10 genotypes. *, ** and *** significant at the 0.05, 0.01 and 0.001 levels; ns, not significant by ANOVA, GF denotes grain filling. For real time expression and NUE indicators, recommended N situation was taken as control and low N was considered as treated for calculations.

reported (Artacho et al., 2009; Geoffrey et al., 2012; Gueye and Becker 2011; Kanfany et al., 2014; Metwally et al., 2010; Singh et al., 1998; Sui et al., 2013; Tirol-Padre et al., 1996; Yoshinaga et al., 2013; Zhang et al., 2013). Thus the panicle number is apparently correlated with the tiller number, however the increase of the number of tillers do not always result in the increase of the number of panicles. Excess tillers have been reported to cause small panicles, poor grain filling and a consequent reduction in grain yield (Peng et al., 1994).

Rice grain yield is mostly decided by the total number of fertile and sterile spikelets (Matsushima 1970, Yoshida et al., 1981). N status of plant during late spikelet differentiation stage decides the number of spikelets (Kamiji et al., 2011). The total number of spikelets per panicle was reduced by 17% under low N in comparison with recommended N condition in the present study. Significant increase of spikelets per panicle with the increase of N application was also reported (Mae et al., 2006; Singh et al., 1998; Sui et al., 2013; Yoshinaga et al., 2013; Zhang et al., 2013). The spikelet number per panicle was determined primarily by the number of differentiated spikelets and secondarily by the number of degenerated spikelets (Hoshikawa, 1989). Thus nitrogen is needed for the development of spikelets and later on for the reduction of the spikelet degeneration. With the lack of nutrients, especially nitrogen, spikelet degeneration becomes more severe. The spikelet production and N application in late spikelet differentiation stage found to be strongly interrelated (Kamiji et al., 2011; Kobayasi et al., 2001a).

Grain number per panicle was reduced by 12% in genotypes under low N condition in comparison to recommended N. In comparison with zero N application, aromatic cultivars have shown 21.2% increase of grains/panicle with 40 kg N ha^{-1} (Mahajan et al., 2010). The decrease in the amount of metabolites under low N could be responsible for the lower number of filled grains per panicle (Metwally et al., 2010). Interestingly, the grain filling percentage has increased under low N conditions in comparison with recommended nitrogen by 6.4% confirming some of the earlier similar observations (Artacho et al., 2009; Geoffrey et al., 2012; Hasegawa, 2003; Kanfany et al., 2014; Mahajan et al., 2010; Mahajan et al., 2012; Sui et al., 2013; Zhang et al., 2013). However significant decrease in spikelet fertility % under low N for medium duration varieties and no significant response for long duration varieties was reported by Singh et al. (1998).

Analysis of panicle topology has shown 15% decrease in spikelets and grains on primary branches of upper portion of the panicle under low N. Around 24% and 17% decreases have been observed in spikelets and grains on secondary branches of the upper portion of the panicle. There was a decrease of spikelets by 16% and grains by 12.4% for the primary branches of lower portion of the panicle. There was a decrease of spikelets by 15.3% and grains by 7.7% for the secondary branches of lower portion of the panicle. However the grain filling percentage has increased by 1.5% for primary branches and 8% for secondary branches of the upper portion of the panicle and by 4.2% for primary branches and 5.6% for secondary branches of the lower portion of the panicle. There was an overall reduction of number of spikelets and grains across the panicle and the spikelets on the secondary branches of the upper portion of the panicle were affected more. The yield increase in high yielding varieties of rice was attributed to the increase of the grain number on secondary branches and the number of spikelets on secondary branches is dependent on the nitrogen fertilization (DRR, 2008; Yamagishi et al., 2002), thus the observation of reduction of spikelets on secondary branches under low nitrogen highlights the importance of panicle topology studies for NUE.

Less than 1% decrease was found in the mean 1000 grain weight of genotypes grown under low nitrogen in comparison to the recommended nitrogen application. No significant change was earlier reported for 1000 grain weight in some studies under low nitrogen and the reason

ascribed was grain weight being a very stable varietal character with spikelet size rigidly controlled by hull size (Gueye and Becker 2011; Hasegawa, 2003; Kanfany et al., 2014; Singh et al., 1998; Tirol Padre et al., 1996; Yoshinaga et al., 2013; Zhang et al., 2013). Contrastingly, N application has been reported to be associated with decrease in 1000 grain weight (Metwally et al., 2010; Qiao et al., 2013). The decrease could be because of the higher number of spikelets per panicle with increased N.

Though considerable decrease was not observed in yield components like grain weight, grain filling percentage, or the components of the panicle, the composite yield parameters like productivity per day and grain yield were found to be reduced by ~40% in the present study under low N. Up to 64.2% increase of grain yield was reported with 40 N kg ha^{-1} in aromatic cultivars in comparison with zero application of N (Mahajan et al., 2010). Grain yield was reported to be positively correlated with number of panicles and spikelets per unit area (Sui et al., 2013). Thus the reduction of panicle numbers per unit area could have drastically reduced the yield under low N situation and further reduction of spikelets per unit area also reduced the yield. (Anzoua et al., 2010; Artacho et al., 2009; Kanfany et al., 2014; Mae et al., 2006; Metwally et al., 2010; Samonte et al., 2006; Sui et al., 2013; Tirol-Padre et al., 1996; Yoshinaga et al., 2013).

About 46% reduction was noted in straw yield. N application was reported to contribute to grain yield more by enhancement of dry matter accumulation than by greater partitioning of dry matter to grains (Mahajan et al., 2012) and zero N application showed a constraint for the above ground dry matter accumulation as well (Qiao et al., 2013). Thus, with the drastic reduction of biomass under low nitrogen, the immediate dependent parameter viz., yield and its dependent components are also being reduced. However the magnitude is not being reflected at the panicle level yield components because the panicle number was already reduced along with the number of spikelets under low N. The expressed yield components like 1000 grain weight and % spikelet fertility are not reduced to that extent as the available spikelets are already less (Artacho et al., 2009; De Datta et al., 1990; Gueye and Becker 2011; Inthapanya et al., 2000; Mae et al., 2006; Singh et al., 1998; Tirol-Padre et al., 1996). If two critical yield components viz., spikelets and panicles are less in number under N, the trade-off for 1000 grain weight and % spikelet fertility appears to be less.

More than 50% reduction of mean total nitrogen content in grains, straw and total biomass was observed in genotypes under low N in comparison with recommended N. There is reduction of N% in grain by 13% and straw 15%. There were reports of no significant reduction of straw nitrogen % by Tirol Padre et al., 1996. The nitrogen content in grain and straw and their association to biomass or yield appears to highly variable across the locations, genotypes, duration of the genotypes, stage of application and sampling (Artacho et al., 2009; De Datta et al., 1990; Gueye and Becker 2011; Inthapanya et al., 2000; Ntanos and Koutrobas 2002, 2003; Li et al., 2014; Mae et al., 2006; Samonte et al., 2006; Singh et al., 1998; Sui et al., 2013; Ying et al., 1998; Yoshinaga et al., 2013; Zhang et al., 2009; Zhao et al., 2012).

The growth period of the ten genotypes ranged from 100 to 140 days under recommended N condition and under low N condition, the total growth period was reduced from 2 to 10 days in comparison with recommended N condition across the genotypes. Since the growth period of genotypes was different, per day productivity was calculated and more than 40% reduction was observed in per day productivity under low N condition.

In the present study, Agronomic Efficiency (difference of grain yield between low and recommended N/N applied) ranged from 8.4 to 27.8 kg kg^{-1} with a mean of 20.5 kg kg^{-1} confirming to the reported range of Agronomic Efficiency as 15 to 25 kg grain produced per kg of applied N of in lowland rice in the tropics (Yoshida et al., 1981) and 19 kg grain per kg N in medium duration and 20 kg grain per kg N in long duration varieties (Singh et al., 1998) reported. Physiological efficiency (difference of biological yield between low and recommended N/difference of N content between low and recommended N) ranged 89.2 to 124 kg kg^{-1} (mean 106.7 kg kg^{-1}) and the values of Physiological Efficiency were slightly lower than the reported values (Fageria and Baligar, 2003). Agro-Physiological Efficiency (difference of grain yield between low and recommended N/difference of N content between low and recommended N) ranged from 24.6 to 63.1 kg kg^{-1} (mean 51.7 kg kg^{-1}). Earlier, Singh et al. (1998) observed Agro-Physiological Efficiency of 64 kg grain produced per kg of N uptake. Apparent Recovery Efficiency (difference of biological yield between low and recommended N/N applied) ranged from 33.0 to 56.9% (mean 41.6%) similar to the reported mean ARE of 39% (Fageria and Baligar, 2003) and 43% of mean Apparent recovery of applied N (AR) % in medium duration and 36% in long duration varieties (Singh et al., 1998). As percentage of N recovery depends on soil properties, methods, amount and timing of fertilizer applications, genotypes and management practices, it was reported to be in the range of 30 to 50% in the tropics (Prasad and Datta 1979). Utilization efficiency ranged from 32.9 to 51.7 kg kg^{-1} with a mean of 43.4 kg kg^{-1}. In tropics, the average utilization efficiency was recorded as 50 kg kg^{-1} in lowland rice, while under Brazilian conditions the mean UE was reported to be 58 kg kg^{-1} (Fageria and Baligar, 2003, Yoshida et al., 1981). With proper N management, up to 53 kg grain per kg N uptake of N physiological efficiency was indicated as achievable target (Peng et al., 1996). Though the nomenclature of NUE indicators similar viz., agronomic nitrogen use efficiency, physiological use efficiency etc., the definitions and parameters used in the various reports are variable (Artacho et al., 2009; Jiang et al., 2004; Li et al., 2014; Mahajan et al., 2010; Mae et al., 2006; Metwally et al., 2010; Samonte et al., 2006; Singh et al., 1998; Sui et al., 2013, Tirol-Padre et al., 1996; Ying et al., 1998). Thus comparisons could be done with only with studies with similar calculations.

The role of NUE was obvious when two genotypes with grain yields those were not significantly different and similar duration under recommended N have shown significantly different total N content e.g. the nitrogen uptake of Tulasi was 26% more than that of Aditya. Thus, some genotypes take more nitrogen to give similar yield response in comparison with other genotypes suggesting the existence of genotypic variation for NUE. Genotypes with higher N content do not have always the highest yield (Gueye and Becker, 2011).

3.2. Differential expression of OsSPL14 gene under low N conditions

Differential expression was not observed in the amplified products of *Os SPL14* gene using regular reverse transcription (RT) PCR resolved on 2% agarose gel (Supplementary Fig. 1). Using real time RT-PCR, the expression levels of *OsSPL14* were studied in leaf and panicle tissues taking expression levels under recommended N as the control (Fig. 1 and Fig. 2). *OsSPL14* was upregulated in the leaf samples of KRH2, Rasi, Tellahamsa and Varadhan (Fig. 1a). The transcripts were reported to be relatively less in leaf sample of transgenic Near Isogenic Line (NIL) with *OsSPL14* of Nipponbare in comparison with shoot apices and culms (Jiao et al., 2010) in normal fertilization conditions. The lower expression of *OsSPL14* in leaves in comparison to panicle tissues was also reported in Nipponbare under normal conditions (http://ricexpro.dna.affrc.go.jp/; http://rice.plantbiology.msu.edu). Interestingly, around fourfold increase of *OsSPL14* in leaf sample under low N condition was observed in genotypes, KRH2, a hybrid (Hari et al., 2011) and Rasi, a variety known for its good grain filling (Subhakara Rao et al., 2011). In the comparative analysis of microRNAs associated with low N tolerance also, enhanced expression of miRNA156 targeting *OsSPL14* gene in leaf sample was observed in low N sensitive genotype (Nischal et al., 2012). Up-regulation of miRNA156 targeting mRNAs encoded by the Squamosa Promoter Binding protein (SBP) or SBP-like (SPL) gene family regulating flowering, vegetative phase change, fertility, and leaf formation during nitrogen starvation has been earlier reported (Fischer et al., 2013; Xie et al., 2006). Though the real time expression of yield related genes was not studied earlier in leaf samples under differential nitrogen

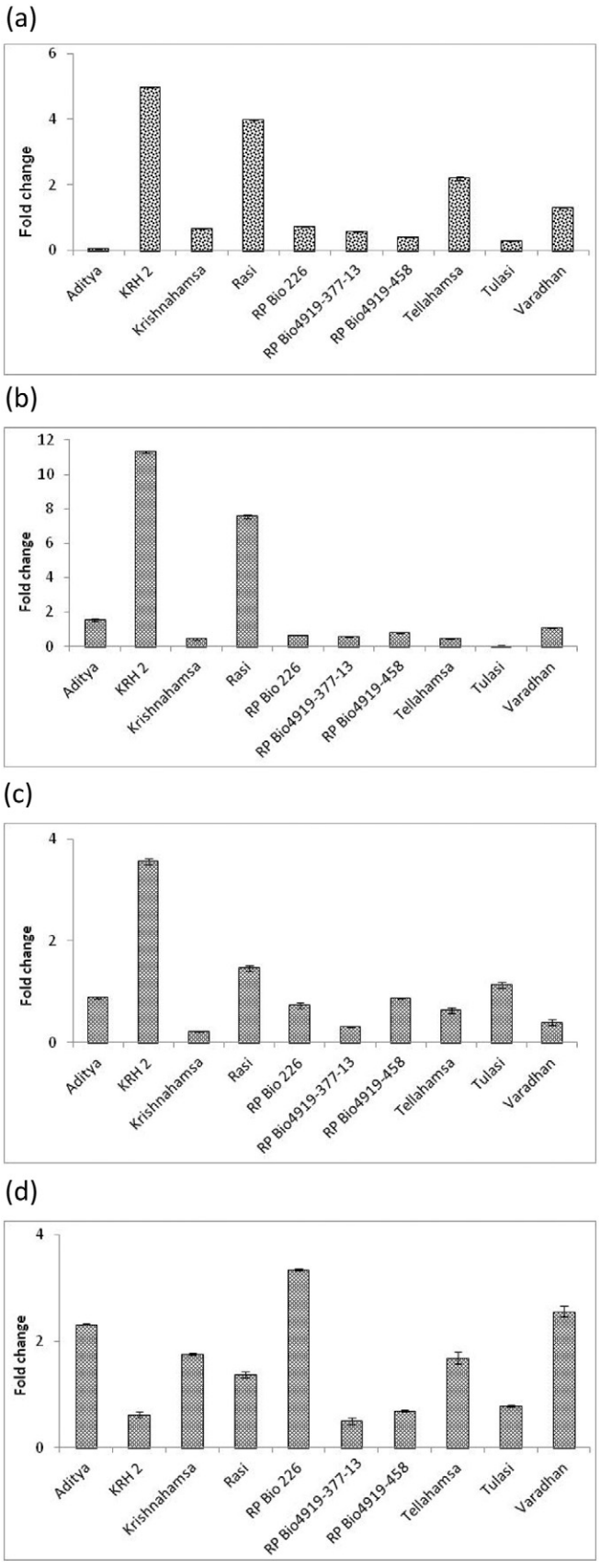

Fig. 1. Graphs showing differential expression of *OsSPL14* in leaf (a), panicle stage 1(b), panicle stage 2(c) and panicle stage 3(d). Increased fold change of *OsSPL14* in low N was derived with recommended N situation as control and low N as treated.

situations, the expression studies of many nitrogen metabolism genes have shown enhanced expression under low nitrogen. Nitrate reductase activity was reduced in nitrogen sensitive cultivar but not changed in NUE rice cultivar under low nitrogen supply under hydroponics (Fan et al., 2007). Enhanced expression of AMT1;1 and OsNRT2;1 under low nitrogen have also been shown demonstrated in rice seedlings hydroponics (Shi et al., 2010). Differential expression of nitrate reductase and glutamine synthetase between low and high nitrogen use efficient cultivars indicated the enzymes to be involved in efficient uptake and utilization (Hakeem et al., 2012). Activities of glutamine synthetase (GS), glutamate synthase (GOGAT) and Glutamate Dehydrogenase (GDH) in a rice hybrid under five nitrogen treatments and three irrigation regimes suggested ammonium assimilation enzyme activities to be associated with grain yield or NUE (Sun et al., 2012).

Increased expression of *OsSPL14* was found in the panicle tissue (stage 1) of Aditya, KRH2, Rasi, and Varadhan (Fig. 1b and Fig. 2); in the panicle tissue (stage 2) of Aditya, KRH2, Rasi, RPBio4919- 377-13 and Tulasi (Fig. 1c and Fig. 2), and in the panicle tissue (stage 3) of Aditya, KRH2, Krishnahamsa, Rasi, RP Bio 226, RP Bio 4919- 377-13 (Fig. 1d and Fig. 2). The genotypic, temporal and spatial differential expression of *OsSPL14* suggests the complexity of the gene and its function (Lu et al., 2013). The increased expression of *OsSPL14* in panicle tissues under recommended fertilizer conditions has been reported in several studies (Jiao et al., 2010; http://ricexpro.dna.affrc.go.jp/; http://rice.plantbiology.msu.edu). In the present study, we are reporting the enhanced expression of *OsSPL14* in panicle and leaf tissues under low nitrogen. Earlier, increased activities of enzymes related to yield components viz., sucrose synthase and adenosine diphosphoglucose phyrophosphorylase (AGPase) were reported under differential N treatments (Zhang et al., 2013). Recent report of the enhanced expression of DEP1 gene regulating rice panicle architecture in NIL-dep1-1 under low nitrogen conditions suggests the possible involvement of the enhancement of *OsSPL14* expression under low nitrogen as observed in the present study. It has been also shown earlier the involvement of *OsSPL14*/IPA1 in positive regulation of DEP 1 controlling panicle architecture (Lu et al., 2013).

The only hybrid in the study, KRH2 has shown increased expression of *OsSPL14* in leaf as well in three stages of panicle. The reported RNA seq transcriptomics of hybrids and their parents showed low or no expression of *OsSPL14* in the leaf samples and low parent level of expression in root and meristems of F_1 hybrid of Nipponbare and 9311 under recommended fertilizer situation (Venu et al., 2014). The observation of enhanced *OsSPL14* expression in rice hybrid needs to be studied further involving more hybrids along with parental lines.

3.3. Sequencing of OsmiR156 motif region

Sequencing of 450 bp PCR product encompassing OsmiR156 motif has not shown any nucleotide polymorphisms in OsmiR156 target in the 10 genotypes of the present study (Supplementary Fig. 2). The sequence of OsmiR156 motif of ten genotypes of the present study was not matching with the reported sequences of OsmiR156 associated with higher panicle branching and grain productivity or ideal plant architecture as reported by Jiao et al., 2010 and Miura et al., 2010. All the 10 sequences of OsmiR156 were matching with the sequences of TN1 and Nipponbare. And TN1 was released as high yield variety with nitrogen responsiveness. Study of Miura et al. (2010) showed that *OsSPL14* gene along with its regulatory elements also harbors several other polymorphisms responsible for higher grain productivity in addition to the single nucleotide polymorphism identified in OsmiR156 motif. Thus the differential expression as observed in the present study warrants a detailed characterization of the complete OsSPL14 gene along with its regulatory elements and its association with NUE. Similar observations were reported in case of DEP1 (Dense and Erect Panicle), a cloned yield gene associated with rice panicle architecture under differential nitrogen doses and different DEP1 alleles

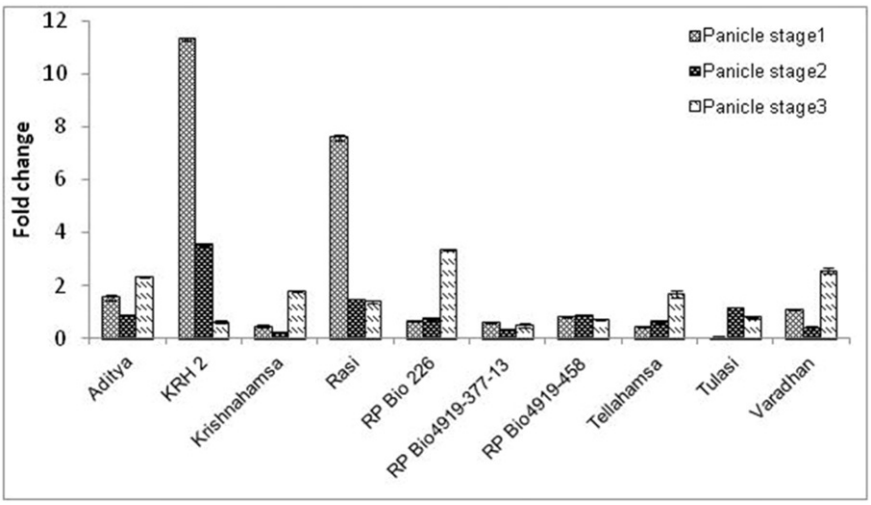

Fig. 2. Graph showing differential expression of *OsSPL14* in three panicle stages 1, 2 and 3 of ten genotypes. Increased fold change of *OsSPL14* in low N was derived with recommended N situation as control and low N as treated.

differentially affect nitrogen-responsive cell proliferation and organ size at different developmental stages (Sun et al., 2014).

3.4. Correlation studies

Under low N, tillers m^{-2} have shown significant association ($P < 0.01$) with panicle m^{-2}. Interestingly, straw yield m^{-2} has shown significant correlation with grain yield (Table 2). Zhao et al. (2012) also found significant correlation between grain yield and straw weight under non-basal N fertilizer application. Under low N, significant correlations were observed for Utilization Efficiency with Agronomic Efficiency and Apparent Recovery Efficiency suggesting the importance of all the parameters viz., grain yield, straw yield, nutrient applied and nitrogen content of straw and grain in deciding NUE of a genotype. At the panicle level, under low N, apart from the obvious significant correlations among spikelets and grains, significant correlations were found between total grain filling (%) with grain filling (%) of secondary branches of upper portion of the panicle and total grains and spikelets with spikelets and grains of secondary branches of lower portion of the panicle. Thus the secondary branches play a critical role for the total grains under low N as well. Out of correlation among yield parameters and NUE indicators studied, 1000 grain weight found to be associated with Agronomic Efficiency (grain yield and nutrient applied) ($P < 0.05$) and Agro-Physiological Efficiency (grain yield, nitrogen content in biomass) ($P < 0.01$). Nitrogen content (%) in straw has shown ($0.05 < P$) with Agro-Physiological Efficiency. Straw N% also showed significant correlation with grain N Use Efficiency (grain yield/total N uptake) earlier under non-basal N fertilizer conditions (Zhao et al., 2012). Nitrogen is significantly correlated with grain yield, panicle mass, panicle density, grain mass and Nitrogen Translocation Ratio (Samonte et al., 2006). NUE was reported to be not associated with yield (Hasegawa, 2003). Significant correlations have also been observed with total grain filling (%) and grain filling (%) of secondary branches of upper portion of the panicle with Apparent Recovery Efficiency ($P < 0.01$). Thus, the correlations of yield and NUE parameters appear to be variable in relation to the earlier studies of correlations of yield parameters suggesting that correlations change considerably with the nitrogen dosages and varieties (Sui et al., 2013; Zhang et al., 2013) (Supplementary Table 2).

The expression of *OsSPL14* at panicle stage 3, has shown correlation ($P < 0.05$) with N% in grain. The importance of maintenance of source activity and translocation of nitrogen during the late ripening period for better yields was earlier underscored in rice (Ida et al., 2009), which was also supported by the observations of ample nitrogen uptake during the heading stage to meet the nitrogen and carbohydrate demands of the panicles (Sheehy et al., 1998; Zhang et al., 2007).

Individual analysis of genotypes has shown enhanced expression of *OsSPL14* in KRH2 and Rasi in leaf and three stages of panicle with difference in magnitude. KRH2 has shown maximum Use Efficiency among the genotypes though not ranked first in the yield and yield related parameters. The hybrid nature of KRH2 could have also played the role. Differential nitrogen Uptake Efficiency of a rice hybrid in comparison of pure-line was demonstrated with differential N soil content (Norman et al., 2013). Rasi has shown enhanced expression *OsSPL14* along with maximum number of tillers, panicles and grain filling of >90% across the panicle under low N (Supplementary Table 1) suggesting the role of *OsSPL14* gene under low N conditions.

Though increased expression in all the three stages of panicle was seen in Aditya, three folds change was observed only in stage 3, thus corresponding to maximum grain filling (%) of spikelets of primary, secondary branches of upper portion of panicle and total grain filling (%) and Agro-Physiological Efficiency. In Varadhan and RP Bio 4919- 377-13, around one fold enhanced expression was only recorded, but Varadhan showed maximum number of spikelets and grains on primary branches of upper portion of panicle and RP Bio 4919-377-13 showed maximum productivity per day, grain yield ($t\ ha^{-1}$) and grains on secondary branches of lower portion of panicle.

Both yield and NUE in rice are complex traits involving several genes and their interactions. Thus, the association of yield under low nitrogen and NUE cannot be explained with a single gene; however we found the association of *OsSPL14* gene with yield component traits under low N in the present study. Earlier it has been shown that translocation of carbohydrates plays an important role in favoring the transport of NADH-dependent GOGAT protein, which in turn favors grain filling (Hayakawa et al., 1993). The recent proof of concept of association of DEP1, a yield component gene with Nitrogen Use Efficiency has substantiated the role of yield genes in NUE in rice (Sun et al., 2014). In rice, grain yield was shown to be significantly correlated with dry matter translocation efficiency and nitrogen translocation efficiency suggesting the role of the sink strength (Ntanos and Koutroubas, 2002). Accumulation and distribution of N in the vegetative and reproductive organs of rice are the important processes in determining grain yield (Norman et al., 1992). Since *OsSPL14* is a functional transcription activator associated with several genes viz., transcription factors, plant hormone related genes, transcriptional regulators, enzymes and other genes (Lu et al., 2013), correlation of the expression of *OsSPL14* gene in leaf and three panicle stages with yield components as observed in

Table 2
Significant correlation of yield components, nitrogen content, real time expression of *OsSPL14* and NUE indicators in low N conditions.

Panicles m^{-2}	Grain yield (t ha^{-1})	Upper primary spikelets	Upper primary grains	Upper primary GF%	Upper secondary spikelets	Upper secondary grains	Upper secondary GF%	Lower primary spikelets	Lower primary grains	Lower secondary spikelets	Lower secondary grains	Lower secondary GF%	Total grains per panicle	Straw yield (t ha^{-1})	N in grain	AE (kg kg^{-1})	ARE(%)
Tillers m^{-2}	0.91***	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Upper primary spikelets	ns	ns	0.95***	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.62*	ns	ns	ns
Upper secondary spikelets	ns	ns	0.65*	ns	ns	0.96***	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Upper secondary grains	ns	ns	0.61*	ns	ns	ns	ns	ns	ns	0.63*	ns	ns	ns	ns	ns	ns	ns
Upper secondary GF%	ns	ns	ns	0.61*	ns	ns	ns	ns	ns	ns	ns	0.70*	ns	ns	ns	ns	0.73**
Lower primary spikelets	ns	ns	ns	ns	ns	ns	ns	ns	0.94***	ns	ns	ns	ns	ns	ns	ns	ns
Lower secondary spikelets	ns	0.60*	ns	ns	ns	ns	ns	ns	ns	ns	0.96***	ns	ns	ns	ns	ns	ns
Lower secondary grains	ns	0.64*	ns	ns	ns	ns	ns	ns	0.62*	ns	ns	ns	ns	ns	ns	ns	ns
Total spikelets	ns	0.65*	0.61*	0.70*	ns	0.73*	0.68*	ns	0.63*	0.62*	0.88***	0.81**	0.95***	0.84**	ns	ns	ns
Total grains	ns	0.64*	ns	0.62*	ns	0.61*	0.63*	ns	0.61*	0.67*	0.94***	0.92***	ns	0.78**	ns	ns	ns
Total GF%	ns	ns	ns	ns	ns	ns	0.90***	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.75**
Straw yield	ns	0.74**	ns	ns	ns	ns	ns	0.66*	ns	0.63*	ns	ns	ns	ns	ns	ns	ns
Productivity per day	ns	0.89***	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.62*	ns	ns
RT-RQ-PS3	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.67*	ns
APE (kg kg^{-1})	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.64*	ns
ARE(%)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.82**	0.78**
UE (kg kg^{-1})	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Only significant correlations were shown. ***significant at $P < 0.001$, **significant at $P < 0.01$, and *significant at $P < 0.05$. GF denotes grain filling..

the present study suggests the differential activities of yield component genes and their role in plant growth and development under differential nitrogen conditions in rice.

4. Conclusion

For cost effective and sustainable rice cultivation, nitrogen use efficiency is one of the current major objectives in rice breeding programs. Ten rice genotypes were grown under low and recommended dose of N application and characterized for yield and yield related parameters and NUE indicators. Significant variation observed for 15 yield related parameters confirming the availability of genotypic variation for yield parameters under low N conditions in rice. Composite yield parameters like productivity per day and grain yield were found to be reduced by ~40% in the present study under low N, though considerable decrease was not observed in yield components like grain weight, grain filling percentage, or the components of the panicle. Promising genotypes with buffer capacity for yield under low N conditions were identified. Correlation analysis of various yield components showed the importance of the secondary branches for the total grains under low N. Enhanced expression of *OsSPL14* (LOC_Os08g39890) gene reported to be associated with increased panicle branching and higher grain yield was observed in panicle and leaf tissues under low nitrogen in some of the genotypes. The expression of *OsSPL14* at panicle stage 3, has shown correlation ($P < 0.05$) with N% in grain. With their complex nature, both yield and NUE though cannot be explained by a single gene, however, the association of *OsSPL14* gene with yield component traits under low N in the present study is encouraging for exploring the possibilities of yield component genes and Nitrogen Use Efficiency in rice.

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Author's contribution

BS carried out the sample collection, cDNA synthesis, physiological and yield parameters collection, carried out the quantitative PCR, and drafted the manuscript. IS helped in sample collection, RNA isolation and cDNA synthesis. KS contributed to the physiological and yield parameters. DS contributed to the data analysis. SRV contributed to writing the manuscript. CNN designed the study, coordinated the work and contributed to writing the manuscript. All authors read and approved the final manuscript.

Conflict of interest

There is no conflict of interest.

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