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High nitrogen use efficiency in rice genotypes is associated with higher net photosynthetic rate at lower Rubisco content

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Abstract

Contrasting rice genotypes differing in leaf mass ratio (LMR) and leaf nitrogen content were screened. A strong inverse relationship was observed between ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) content and its efficiency estimated as the ratio of net photosynthetic rate (P_N) to Rubisco content. Similarly, an inverse relationship between the specific activity of fully activated Rubisco and its content was observed. This suggests that a down regulation of Rubisco may occur if the efficiency of the enzyme is superior. Genotypes IET 12989 and IET 13567 recorded higher P_N together with lower Rubisco content in comparison with other genotypes measured. These genotypes showed low LMR and low nitrogen content and hence could be considered as efficient nitrogen users.

Additional key words: leaf mass ratio, leaf nitrogen content, *Oryza sativa*, Rubisco efficiency.

Introduction

Though the responsiveness of rice plant growth and productivity to nitrogen supply is well documented in the literature, not much has been studied to assess the genetic variability in nitrogen use efficiency (NUE). Maximising the photosynthetic carbon fixation, growth rate, and finally the productivity at low input level of nitrogen is highly relevant in improving the economy of rice cultivation, particularly in semi-arid tropics.

Plants allocate an appreciable proportion (over 35 %) of nitrogen towards a single enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the activity of which controls the rate of carbon fixation (Evans 1989, Makino *et al.* 1992). Any effort to improve the efficiency of this enzyme would enhance the photosynthetic rate besides economising on the over all nitrogen budgeting (Makino *et al.* 1984) and hence would be a plausible approach to increase NUE. Recent advent in the molecular biological approaches has added greater insight into these possibilities. Ishimaru *et al.* (2001) have identified several QTLs controlling Rubisco and soluble protein content in rice. Mapping on a QTL for Rubisco content near the QTL for protein content suggest the possibility of improving NUE.

The total catalytic activity of Rubisco is regulated by

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Abbreviations: c_i - intercellular CO_2 concentration; ELISA - enzyme linked immuno sorbant assay; g_s - stomatal conductance, LMR - leaf mass ratio (leaf dry mass to total dry mass ratio); NUE - nitrogen use efficiency; P_N - net photosynthetic rate; PPFD - photosynthetic photon flux density; QTLs - quantitative trait loci; Rubisco - ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP - ribulose-1,5-bisphosphate; TDM - total dry mass.

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its content, state of activation and kinetics of catalysis (Morrel *et al.* 1992). Higher Rubisco activity can be achieved by altering the activation state or improving the kinetic constants (Sage *et al.* 1988, Ecochard *et al.* 1991). However, a unique association seems to exist between these parameters. Down regulation of Rubisco content in CO_2 enriched plants and enhanced allocation of nitrogen to Rubisco in transgenic plants with an antisense construct of Rubisco activase was demonstrated by Mate *et al.* (1993, 1996). These results substantiate that carboxylation efficiency regulated either by substrate levels or kinetic constants would determine the Rubisco content (Makino *et al.* 1997, 2000). From crop improvement point of view, it would be rewarding to identify genotypes that have high carboxylation efficiency and relatively low Rubisco content.

With this objective, we assessed the genetic variability in net carbon gain per unit leaf nitrogen among a few genotypes of rice. In this paper, we focused on an inverse relationship between Rubisco content and its specific activity with the aim to identify genotypes with maximum productivity under low input conditions.

Materials and methods

Field screening: Thirty eight germplasm lines of rice (*Oryza sativa* L.) were screened for the genetic variability in leaf nitrogen content and photosynthetic activities. This experiment was conducted under fully irrigated field condition at the Regional Research Station of the University of Agricultural Sciences, Mandya, India during the rainy season (July - November). Twenty one-day-old seedlings were transplanted to main field in 4 m rows, and plants were grown as per recommendations. Leaf mass ratio (LMR), total dry mass, grain yield and leaf N content were determined from three sets of replications. Each replication was an average value from three uniform plants selected randomly. Plants were oven-dried at 70 °C for 3 d. However, panicles were removed and air-dried until the seed moisture content reached 14 %.

The leaf mass ratio (LMR) was computed from the ratio of the leaf dry mass to the total plant dry mass. LMR is often considered as a good estimate of net carbon gain. For estimation of leaf N, all the leaves of a plant were pooled and homogenised to a fine powder. A composite sample of 0.5 g from the powder was drawn for N estimation as per Kjeldahl's method (Piper 1957). Based on the differences in LMR and leaf N content, seven contrasting genotypes were selected for further studies.

Container experiment: Seedlings of the selected genotypes were raised at the Department of Crop Physiology, UAS, Bangalore, India. Seedlings were transplanted to suitable containers (30 × 15 × 20 cm) filled with 14 kg of wetland soil. Three hills were maintained in each container with 5 replications. Growth parameters such as leaf area, leaf dry mass, and total dry mass were recorded. A composite sample of the leaf powder was obtained to estimate N content. Gas exchange parameters such as photosynthetic rate (P_N), stomatal conductance (g_s) intercellular CO_2 concentration (c_i), and biochemical traits such as protein content, Rubisco content and total activity of Rubisco were determined for all the genotypes.

Gas exchange parameters were recorded on the third fully expanded leaf from top using a portable photosynthetic system (*LCA-4, ADC,* Hoddesdon, UK) on bright sunny days (PPFD > 1500 μmol m^{-2} s^{-1}). All the observations were taken at constant vapour pressure difference (VPD) of 1.5 MPa between 09:00 and 12:00.

Total soluble protein of leaf was quantified using a simple protein-dye binding method of Bradford (1976), using bovine serum albumin as the standard. One gram of leaf material was homogenised to thin paste and soluble protein was extracted with 10 cm^3 of phosphate buffer (pH 7.8) containing 1 mM EDTA, 2 % (m/v) polyvinylpolypyrrolidone (PVP). The extract was filtered through three layers of cheesecloth and centrifuged at 8 000 *g* for 10 min. The absorbance after adding an aliquot of the extract to the Bradford reagent was recorded at 595 nm using a UV-Vis double beam spectrophotometer (*Genesys™-II, Milton Roy Spectronic,* Rochester, USA).

Polyclonal antibody of Rubisco was developed by immunizing a 30 d-old New Zealand rabbit with 1.2 mg of the lyophilised pure Rubisco dissolved in 0.6 cm^3 of phosphate buffered saline (PBS) (pH 7.4), emulsified with 0.4 cm^3 of complete Freund's adjuvant. Two booster doses of the antigen (0.5 mg Rubisco with Freund's incomplete adjuvant) were given to the animal at 15-d interval. After checking the titre of the Rubisco antibody in rabbit serum by dot blot analysis, the animal was bled by ear vein puncture after 10 d of booster injection (Debabrata Ray 1998). The purified polyclonal antibody worked well at 1:2 000 dilution.

The Rubisco content in the selected genotypes was determined by an enzyme-linked immunoassay technique (ELISA). A standard curve in the range of 10 to 100 ng of pure Rubisco was developed for the quantification of enzyme content in plant samples. A known area of the leaf earlier used for gas exchange measurements was immediately frozen to liquid nitrogen temperature and used for the quantification of Rubisco content and activity. The frozen leaf sample was taken and extracted in three volume of extraction buffer (100 mM Tris-HCl,

pH-8.2, 20 mM KCl, 0.1 mM EDTA, 2 % PVP and 5 mM β-mercaptoethanol). An aliquot of the extract was drawn for quantification of Rubisco content.

For determination of initial activity and total specific activity of Rubisco, an aliquot of cell-free protein extract was immediately assayed in a 100 mM Tris-HCl buffer (pH 8.2) containing 0.5 mM RuBP and $NaH^{14}CO_3$ to determine initial activity. The activity was determined as the amount of radioactivity ($^{14}CO_2$) incorporated in acid stable 3-phosphoglyceric acid (PGA) using a liquid scintillation counter (*Wallac*, Turku, Finland).

Another aliquot of the extract was incubated in the activation buffer containing 100 mM Tris HCl (pH 8.2), 20 mM $MgCl_2$, 20 mM $NaHCO_3$ + $NaH^{14}CO_3$, to fully activate the enzyme. After 10 min of incubation, the enzyme was assayed by initiating the activity by adding 0.5 mM RuBP.

Results

Genetic variability in total dry matter and leaf nitrogen content: Significant genetic difference was noticed in total dry mass, grain yield and leaf nitrogen content (Table 1). The ratio of leaf dry matter to total dry

Table 1. Variability in a few growth parameters, productivity and leaf nitrogen status in 38 genotypes of rice grown under field condition. Total dry mass was quantified on the whole plant basis by taking the oven dry mass (plant samples were oven dried at 70 °C for 3 d). Leaf mass ratio is the ratio of total leaf dry mass to the whole plant dry mass. Leaf N content was quantified by employing the Kjeldahl's method (Piper 1957).

Parameter	Range	Mean	$CD_{0.05}$
Total dry mass [g plant^{-1}]	19.02 - 44.01	3.61	2.223
Leaf mass ratio	0.07 - 0.18	0.13	0.012
Leaf nitrogen content [%]	1.15 - 1.68	1.36	0.052
Grain yield [g plant^{-1}]	8.37 - 17.86	12.16	4.110

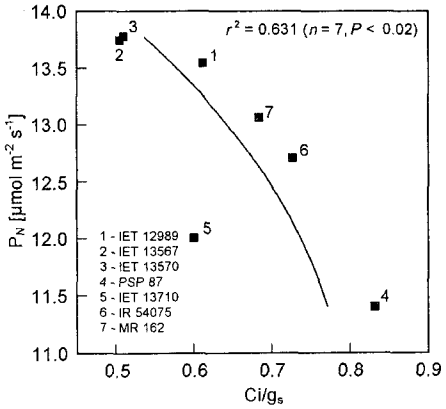

Fig. 1. Relationship of net photosynthetic rate (P_N) with mesophyll efficiency (Ci/g_s) in seven selected genotypes of rice. Gas exchange parameters were taken on top 3rd fully expanded leaf at the PPFD of 1500 µmol $m^{-1}s^{-1}$. Intercellular CO_2 concentration (C_i) at a given g_s was computed as a measure of mesophyll efficiency.

Table 2. Genetic variability in a few growth parameters, gas exchange traits and parameters associated with Rubisco in the selected rice genotypes differing in LMR and leaf N content. TDM - total plant dry mass [g plant^{-1}]; LMR - leaf mass ratio; N - leaf N content [%]; P_N - net photosynthetic rate [µmol(CO_2) m^{-2} s^{-1}]; g_s - stomatal conductance [mmol m^{-2} s^{-1}]; C_i - intercellular CO_2 concentration [cm^3 m^{-3}]; C_i/g_s - a reflection of mesophyll efficiency; Rub - Rubisco content [g m^{-2}(leaf area)]; Prot - protein content [g m^{-2}(leaf area)]; R/P - Rubisco to soluble protein ratio [%]; P_N/R - *in vivo* efficiency of Rubisco [µmol g^{-1}]; sp. act. - specific activity of fully activated Rubisco [Bq mg^{-1}(Rubisco)]; AS - activation state of Rubisco [%].

Genotype	TDM	LMR	N	P_N	g_s	C_i	C_i/g_s	Rub	Prot	R/P	P_N/R	sp. act.	AS
Low LMR and Low N types													
IET 12989	19.81	0.331	1.70	13.54	350	215	0.614	0.37	1.30	32.90	36.59	883.15	50.0
IET 13567	17.39	0.428	2.10	13.75	430	218	0.507	0.44	1.57	28.00	31.25	854.26	72.5
Low LMR and High N types													
IET 13570	19.10	0.371	2.35	13.78	430	221	0.512	0.92	2.46	37.60	15.00	695.37	43.6
High LMR and Low N types													
PSP-87	14.68	0.626	1.88	11.4	240	200	0.833	0.48	1.80	26.75	23.75	631.67	38.0
IET 13710	14.36	0.506	1.98	12.00	410	247	0.602	0.51	1.80	28.50	29.51	757.41	47.7
High LMR and High N types													
IR54075	15.80	0.487	2.24	12.70	310	227	0.728	0.52	2.00	26.35	24.42	712.41	68.7
MR 162	17.37	0.461	2.77	13.06	340	234	0.685	0.75	2.52	29.75	17.41	703.52	45.7
$CD_{0.05}$	3.02	0.046	0.13	0.68	68	13	-	0.08	0.31	-	-	35.19	15.1

matter (leaf mass ratio, LMR) varied significantly among the genotypes. Seven contrasting genotypes differing in LMR and leaf nitrogen were selected for further studies. The low-LMR types recorded relatively higher total biomass than the high-LMR types (Table 2). In addition, both low- and high-LMR types showed distinct variation in leaf N content (Table 2).

Gas exchange: The selected genotypes differed significantly in gas exchange parameters like photosynthetic rate (P_N), stomatal conductance for CO_2 transfer (g_s), and intercellular CO_2 concentration (c_i) (Table 2). The low-LMR types recorded higher P_N than the high-LMR types. A significant inverse relationship between P_N and LMR ($r^2 = 0.45$; $P < 0.05$, $n = 7$) suggested that the LMR could be considered as an

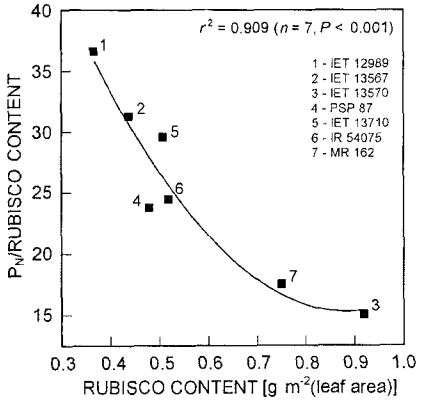

Fig. 2. Relationship of Rubisco content with *in-vivo* Rubisco efficiency (P_N/Rubisco) in seven selected genotypes of rice. Rubisco content was quantified by indirect ELISA technique. The net photosynthetic rate (P_N) to Rubisco contents is considered as a measure of *in-vivo* Rubisco efficiency.

indirect estimate of P_N. We provided experimental evidences to a hypothesis that the ratio of c_i to g_s is a fairly good estimator of the mesophyll efficiency of carbon fixation (Sheshshayee *et al.* 1996). The differences in c_i can be brought about by either g_s or the mesophyll efficiency to consume the substrate CO_2. Any difference in the c_i at a given g_s should therefore reflect the mesophyll capacity. A strong inverse relationship between P_N and c_i/g_s (Fig. 1) indicates that genetic variability in P_N was also brought about by differences in the factors associated with g_m.

Discussion

Agronomic inputs like water and nitrogen are by far the most important limiting factors for realising the potential growth rates in semi-arid tropical climates. The major approach has therefore been to identify the genotypes with higher net carbon gain per unit leaf nitrogen.

Rubisco content and its activity: The genotypes differed significantly in the amount of total soluble proteins, and Rubisco content and its specific activity if fully activated (Table 2). The genotypes that recorded high leaf N status consistently showed higher total soluble proteins and Rubisco content. Although there was a considerable genetic difference in the proportion of the total soluble proteins allocated to Rubisco (Table 2), a positive relationship between protein content and Rubisco was noticed ($r^2 = 0.846$; $P < 0.001$; $n = 7$). However, no significant relationship between P_N and Rubisco content could be established, but the trend was positive.

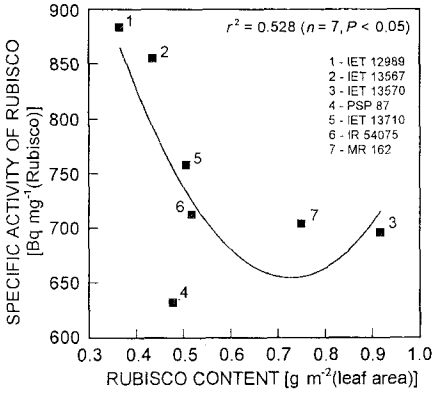

Fig. 3. Relationship of Rubisco content with Rubisco specific activity in seven selected genotypes of rice. Total activity Rubisco was determined after complete activation of the enzyme by incubating it in buffer containing 100 mM Tris-HCl, (pH 8.2) 20 mM $MgCl_2$, 20 mM $NaHCO_3$, $NaH^{14}CO_3$ (3.7×10^4 Bq) for 10 min.

We computed the ratio of P_N to Rubisco content (P_N/Rubisco) as an indirect estimate of *in-vivo* carboxylation efficiency. The P_N/Rubisco, though differed among the genotypes, no discernible relationship was noticed with P_N. Interestingly, a significant inverse relationship was observed between Rubisco content and its *in-vivo* carboxylation efficiency (Fig. 2). To test this further, the specific activity of the fully activated enzyme was determined (Table 2). A similar inverse relationship between Rubisco content and the specific activity (Fig. 3) was noticed. Since, both Rubisco content and its specific activity control P_N, it is possible that the genotypes with higher efficiency of Rubisco could significantly economise on the allocation of soluble proteins to Rubisco without an appreciable reduction in P_N.

In our results, genetic variability in leaf nitrogen status and leaf mass ratio was confirmed in 38 germplasm lines of rice (Table 1). The contrasting genotypes selected based on differences in LMR and leaf N showed a consistent trend in gas exchange parameters (Table 2).

Though stomatal control of the variability in P_N was evident, a significant relationship between P_N and the C_i/g_s ratio (Fig. 1) suggested a considerable mesophyll control of P_N (Sheshshayee *et al.* 1996, Krishnaprasad *et al.* 1996). Significant genetic differences in the parameters that most regulate the carbon fixation, viz., Rubisco content and the specific activity of the fully activated enzyme was noticed among the selected genotypes (Table 2). Despite a considerable genetic variability in the amount of soluble protein allocated to Rubisco, total soluble protein and Rubisco contents were strongly related ($r^2 = 0.84$; $P < 0.05$).

It was interesting to notice an inverse relationship between the *in vivo* efficiency of Rubisco (P_N/Rubisco) and its content (Fig. 2). This initially suggested that if the efficiency of the enzyme is high, the Rubisco content could be considerably reduced without an appreciable reduction in P_N. A significant inverse relationship between Rubisco content and the specific activity of the fully activated enzyme (Fig. 3) further proves that genotype could fix as much CO_2 with reduced Rubisco content if the efficiency of the enzyme is higher. Several authors have also made similar inferences with different systems. In plants acclimated to elevated CO_2 in long-term experiments, a significant reduction in Rubisco content and leaf nitrogen content has been reported (Besford *et al.* 1989, Nakino *et al.* 1997). In such situations, higher substrate availability is known to maintain P_N (Stitt 1991, Sage 1994, Stitt and Krapp 1999). On the one hand, prolonged exposure of plants to elevated CO_2 has also been shown to result in decreased levels of transcripts for proteins necessary for photosynthesis (Nie *et al.* 1995, Chung *et al.* 1998, Moore *et al.* 1998). This is perhaps one of the reasons for acclimation of photosynthesis to long-term exposure to elevated CO_2. On the other hand, Mate *et al.* (1993) reduced the activation of Rubisco through a reduction in Rubisco activase content using antisense technology. They noticed a significant increase in Rubisco content, which was almost completely responsible for the observed increase in soluble protein content. In our studies too, we found that genotypes that have higher efficiency of Rubisco may significantly down regulate the synthesis of Rubisco and hence would be efficient users of nitrogen nutrition.

In summary, the present findings establish that, genotype with low nitrogen content and high P_N sustained high carbon gain by virtue of higher Rubisco activity than by content. The nitrogen thus saved could substantially increase NUE. Since Rubisco plays a pivotal role in regulating the nitrogen economy, identification of such genotypes would have a distinct advantage in breeding programmes. The genotypes IET 12989 and IET 13567 showed reduced Rubisco content but had the ability to produce higher biomass per unit amount of leaf nitrogen and could be considered as efficient nitrogen users.

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