Intraspecific variation in photosynthetic nitrogen-use efficiency is positively related to photosynthetic rate in rice (*Oryza sativa* L.) plants

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Abstract

To explore the natural variation in leaf photosynthesis and investigate its relations with leaf nitrogen (N) status, 121 rice cultivars were pot-grown and their leaf photosynthetic rate (P_N) and N content were measured on the flag leaves at heading stage before anthesis. The results showed that there were substantial natural variations in P_N, leaf N content based on leaf area and leaf mass, and photosynthetic N-use efficiency (PNUE). P_N ranged between 15.4 and 32.6 µmol m^{-2} s^{-1}; P_N and PNUE of four widely grown cultivars in China were significantly lower than P_N and PNUE of the cultivar Nancay PA, which possessed the highest values for P_N and PNUE. Moreover, PNUE was positively related to P_N across cultivars, the high PNUE was related to high stomatal conductance, high transpiration, and low leaf mass per area. We concluded that intraspecific variation in PNUE is positively related to P_N in rice plants.

Additional key words: CO_2 diffusion conductance; leaf anatomy; leaf hydraulic conductance.

Introduction

Improvements in crop management and genetic gain in harvest index lead to a dramatic increase in crop yields, which have more than doubled since 1960 (Long *et al*. 2015). Although the yields continue to increase in most growing areas on a global scale, the yield improvements are stagnating or even moving in reverse at about 24–39% of the growing areas for maize, rice, wheat, and soybean crops (Ray *et al*. 2012, Long 2014). Ray *et al*. (2013) found that the global yields of these four major crops are now increasing at a rate of 1.6, 1.0, 0.9, and 1.3% per year, respectively, which is far less than the rate of 2.4% per year required to double global production by 2050. Therefore, a second Green Revolution is needed to boost crop production and to meet the rising demand.

Over the past 50 years, intercepted radiation and the harvest index have been largely improved, but the conversion efficiency of visible solar energy, which is related to photosynthesis and respiratory loss, has not been improved (Long *et al*. 2015). Thus, increasing energy conversion efficiency can promote the yield.

Many researches have aimed to select genotypes for high photosynthesis and were successful in identifying such cultivars (Moss and Musgrave 1971, Austin *et al*. 1982, Shibles *et al*. 1987), but most of these cultivars could not reach the higher crop yield (Moss and Musgrave 1971, Ford *et al*. 1983). The main reason is the complexity of relations between instantaneous assimilation rate of a single leaf and the biomass/yield at the level of a crop canopy. The yield formation is a complex process, the relationship between yield and photosynthesis of a single leaf may be influenced by many factors, such as respiration rate, transportation, *etc*. In addition, Sinclair *et al*. (2004) illustrated that the impact of factors that underlie crop yield can be diminished when scaling up to a higher level, *e.g*., a 50% increase in mRNA synthesis for Rubisco carboxylase finally increases (or even decreases) yield by up to 6% depending on the level of nitrogen accumulation. Therefore, though many previous researches showed weak relations between the yield and net photosynthetic rate (P_N), improving P_N and ascertaining its mechanism is necessary for future breeding work.

There are many approaches to enhance P_N, for example, designing more efficient Rubisco, increasing mesophyll conductance (g_m), and introducing the C_4 photosynthetic pathway into C_3 crops (Long *et al.* 2006). Suzuki *et al.* (2007) have increased the Rubisco content in rice leaves using transgenic methods, but it failed to enhance P_N due to the changes in N distribution. Introduction of C_4 photosynthetic pathway into C_3 crops seems to be an attractive and promising way to increase P_N, however, it can take several decades to achieve this ambitious aim due to difficulties in changing leaf anatomy (as C_4 plants have special Kranz anatomy) and the complex biochemical actions. Therefore, other strategies should be considered to increase P_N at present.

In addition to these genetic approaches, exploiting natural variation in leaf photosynthesis can also contribute to increasing crop productivity, *e.g.*, rice production (Gu *et al.* 2014). Using the crop model GECROS (Yin and van Laar 2005), Gu *et al.* (2014) showed that improving P_N by exploiting its genetic variation in rice plants can effectively increase biomass production, it shows that an increase in P_N by 25% can lead to an increase in biomass production of 22–29%. Natural genetic variation is the fuel of evolution, and is a prerequisite for natural or artificial selection (Flood *et al.* 2011). However, the natural variation in crop photosynthesis has not been fully studied and exploited, as most studies on photosynthesis are conducted with a few varieties (Cook and Evans 1983, Sasaki and Ishii 1992, Yeo *et al.* 1994, Giuliani *et al.* 2013, Xiong *et al.* 2017). Driever *et al.* (2014) showed a large variation of P_N between 20.5 and 31.5 μmol m^{-2} s^{-1} in 64 wheat cultivars. Using 24 rice and wild relatives, Giuliani *et al.* (2013) explored the variations of leaf structure and its relations with photosynthesis and transpiration. In addition, Xiong *et al.* (2017) revealed that leaf anatomy can affect both out-of-xylem hydraulic conductance (K_{ox}) and the mesophyll CO_2 diffusing rate by using 11 *Oryza* genotypes. But studies on the distribution of P_N among widely grown rice cultivars and its relations with leaf N content are lacking. It is not known how large potential still exists for improving photosynthesis in widely grown rice cultivars.

P_N is commonly observed to be positively related to the leaf N content per area (N_{area}), because up to 27% of the total leaf N content is invested into Rubisco (Evans 1989, Makino *et al.* 1997). This suggests that the natural variation in leaf photosynthesis is related to Rubisco and leaf N content (Driever *et al.* 2014), and more leaf N and Rubisco may be required for cultivars possessing a high photosynthetic capacity. Engineering plants with more efficient Rubisco or the introduction of the C_4 photosynthetic pathway into C_3 crops can simultaneously improve P_N and PNUE (Lin *et al.* 2014, Carmo-Silva *et al.* 2015, Long 2015), a major component of N-use efficiency, defined as a ratio of P_N to N_{area}. However, it is not known whether intraspecific variation in PNUE is related to P_N.

In addition to the leaf N content, there are many other factors that can substantially affect photosynthesis in C_3 plants. According to FvCB (Farquhar *et al.* 1980) model of leaf photosynthesis, photosynthesis of C_3 plants can be affected by one of three factors: (*1*) activity of Rubisco; (*2*) ribulose-1,5-bisphosphate (RuBP) regeneration; and (*3*) release of phosphate during the metabolism of triose phosphate to either starch or sucrose (Farquhar *et al.* 1980, Sage 1990, Harley *et al.* 1992, Manter and Kerrigan 2004). Under current ambient conditions [around 400 μmol(CO_2) mol^{-1}(air)], CO_2 diffusion conductance from air to carboxylation site was regarded to be one of the limiting factors for P_N in C_3 plants (Li *et al.* 2009, Yamori *et al.* 2011, Adachi *et al.* 2013, Flexas *et al.* 2012, Evans *et al.* 2009). CO_2 diffusion pathway from atmosphere to chloroplast stroma can be divided into two phases – stomatal diffusion and mesophyll diffusion, the corresponding conductances of these two phases are called stomatal conductance (g_s) and g_m, respectively. The g_s is determined by the stomatal features, especially by stomatal aperture. For the reason that CO_2 and H_2O share a common path to enter and diffuse out the stomata, stomatal aperture is highly correlated with plant hydraulic status, which can be effectively represented by transpiration rate (E) (Brodribb *et al.* 2007, Giuliani *et al.* 2013, Nardini *et al.* 2014). For mesophyll diffusion pathway, CO_2 needs to pass through the mesophyll cell to get to the carboxylation site after reaching substomatal cavities (Terashima *et al.* 2011). Therefore, g_m is greatly determined by leaf anatomy. Leaf mass per area (LMA), the ratio of leaf mass to leaf area, is a parameter which is widely used as a leaf anatomical feature. Many researches have showed that high LMA can result in a low g_m (Hassiotou *et al.* 2009, 2010; Muir *et al.* 2014). Therefore, the effects of g_s, E, and LMA on P_N and PNUE was investigated to reveal the impacts of CO_2/H_2O diffusion efficiency and leaf anatomy on photosynthetic capacity in present study.

In present study, a pot experiment was conducted using 121 rice cultivars. P_N and its related physiological parameters, *i.e.*, leaf N content, were measured on the flag leaves. The objectives were: (*1*) to study natural variation of leaf photosynthesis in rice plants; (*2*) to explore the relationship between P_N and leaf N content across a large group of rice cultivars; (*3*) to investigate the relationship between P_N and PNUE.

Materials and methods

Plant materials and N input: A total of 121 rice cultivars were collected from 13 countries (Table 1S, *supplement*). After germination on moist filters on 11 May 2014, rice cultivars were transferred to nursery plates. When the seedlings developed in average of three leaves, they were transplanted to 11-L pots with a density of three hills per pot and two seedlings per hill. There were five pots per cultivar and each pot was filled with 10 kg of soil, phosphorus (P) and potassium (K) were applied as basal fertilizers at an amount of 1.5 g per pot. N was applied at the amount of 2 g(N) per pot, 40% of which was applied as a basal fertilizer and next two topdressings of 30% each were applied at mid-tillering and the heading stages, respectively. Plants were watered daily, and a minimum 2-cm water layer was maintained to avoid drought stress. Pests were intensively controlled using chemical pesticides. The experiment was

conducted outdoors in Huazhong Agricultural University (114.37°E, 30.48°N) in Wuhan City, Hubei Province, China. Measurements were conducted at the heading stage before anthesis.

Gas exchange was measured with a portable photosynthesis system (*LI-6400XT, LI-COR Inc.*, Lincoln, NE, USA) on the flag leaves between 09:00 and 16:00 h. Throughout all experiments, PPFD during measurements was set to 1,500 μmol(photon) m^{-2} s^{-1} using a red-blue LED artificial light source, with 10% of blue light. Relative humidity was controlled at ~60% to minimize the impacts of environmental fluctuation on g_s and P_N, which resulted in a relative stable VPD of 1.69 ± 0.53 kPa. CO_2 concentration in the leaf chamber was about 393 ± 13 μmol mol^{-1}. Leaf temperature was set to 28°C. Data were recorded after equilibration to a steady state, about 20 min after enclosing the leaves in the leaf chamber.

In order to compare photosynthetic characteristics of leaves with a similar developmental age, gas-exchange measurements of each cultivar were conducted immediately after the flag leaves fully expanded. The measurements lasted for 19 d in July and August, and 5–6 cultivars were typically examined in a single day. The measurements were conducted only on sunny days, during which the radiation intensity, average temperature, and relative humidity were 19.2 ± 4.6 MJ m^{-2} d^{-1}, 29.1 ± 2.4°C, and 77.0 ± 5.8%, respectively (Fig. 3S, *supplement*). Moreover, the environmental conditions in the leaf chamber were tightly controlled by the gas-exchange instrument, which minimized the variations of gas-exchange parameters caused by different ambient environmental conditions. To minimize the diurnal variation of photosynthesis, the measurements were suspended when significant midday depression occurred.

Leaf N content measurements: Leaves were detached immediately after the gas-exchange measurements, followed by leaf area measurement using a *LI-Cor 3000C* (*LI-COR Inc.*, Lincoln, NE, USA) leaf area analyzer. Leaves were then oven-dried at 80°C until they achieved a constant mass. Afterwards, leaf dry mass was weighed, and leaf mass per area was calculated as the ratio of leaf dry mass to leaf area. Mass-based leaf photosynthesis (P_{mass}, μmol g^{-1} s^{-1}) was calculated using the equation:

$$P_{mass} = \frac{P_N}{LMA} \qquad (1)$$

Leaf equation: Leaf N content based on leaf mass (N_{mass}) was measured with an *Elementar Vario MAX CN* analyzer (*Elementar Analysesysteme GmbH*, Hanau, Germany), and N_{area} was calculated by multiplying N_{mass} with LMA. PNUE was calculated using the equation:

$$PNUE = \frac{P_N}{N_{area}} \qquad (2)$$

Statistical analysis: One-way analysis of variance (*ANOVA*) was used to assess varietal differences in each parameter using *Statistix 9* software (*Analytical Software*, Tallahassee, Florida, USA). Linear regression analysis was performed to test the correlations between parameters using *SigmaPlot 10* (*Systat Software Inc.*, California, USA). The normal distribution tests of the various photosynthesis traits were conducted by One-sample Kolmogorov–Smirnov′s test using *SPSS 16.0* (*SPSS Inc.*, Chicago, IL, USA).

Results

N_{area} and N_{mass} showed great variations among 121 rice cultivars (Fig. 1, Table 1S). N_{area} ranged from 0.94 g m^{-2} for cultivar N22 (NO. 108) to 1.99 g m^{-2} for cultivar Belgioioso (NO. 54), with a mean value of 1.51 g m^{-2}. N_{mass} ranged from 1.70% for cultivar Yangdao 6 (NO. 144) to 3.83% for cultivar PI 282203 (NO. 88), with a mean value of 2.78%. Similarly, P_N was highly variable among the 121 rice cultivars, ranged from 15.5 μmol m^{-2} s^{-1} for cultivar N22 (NO. 108) to 32.6 μmol m^{-2} s^{-1} for cultivar Nancay PA (NO. 33), with a mean value of 23.3 μmol m^{-2} s^{-1} (Fig. 2*A*, Table 1S). Differences between cultivars were highly significant. N_{area} of the cultivar with the highest P_N (cultivar 33, Nancay PA) was 1.45 g m^{-2} (N_{mass} = 2.39%; Fig. 1, Table 1S), which was lower than the mean value of 1.51 g m^{-2} (2.78% for N_{mass}). P_N of widely grown rice cultivars in China – Huanghuazhan (NO. 99), Shanyou 63 (NO. 110), Liangyoupeijiu (NO. 107), and Yangliangyou 6 (NO. 118) – were 23.6, 25.0, 25.7, and 26.1 μmol m^{-2} s^{-1}, respectively (Fig. 2, Table 1S). These values were slightly (1.3–12.0%) higher than the mean value of all the 121 cultivars, but were significantly lower (19.9–27.8%) than the highest P_N (32.6 μmol m^{-2} s^{-1}). N_{area} of these four cultivars were 1.55, 1.56, 1.65, and 1.69 g m^{-2}, which were 2.6–11.9% higher than the mean N_{area} of all the cultivars and were 7.9–17.1% higher than cultivar 33 (Fig. 1, Table 1S). N_{mass} were 2.44, 2.59, 2.99, and 2.91%, respectively, which were either lower (for cultivars 99 and 107) or higher (for cultivars 110 and 118) than the mean value of 2.78% in all cultivars. But they were all higher (2.2–24.9%) than that of the cultivar 33.

Across all cultivars, P_N was positively related to N_{area} (Fig. 3*A*), but the correlation coefficient was very low ($R^2 = 0.18$). N_{area} of 1.4 g m^{-2} is usually used as a threshold, below which N fertilization is needed in rice plants (Peng *et al*. 1996). If the correlation between P_N and N_{area} was calculated for cultivars with $N_{area} > 1.4$ g m^{-2}, no significant correlation was observed ($P = 0.1955$, $n = 91$), while it was significant for cultivars with $N_{area} < 1.4$ g m^{-2} ($P = 0.003$, $n = 30$). This suggested variation in P_N, when $N_{area} > 1.4$ g m^{-2} did not result from differences in N_{area} (Table 1).

PNUE was also significantly different between all rice cultivars and ranged from 10.03 μmol(CO_2) g^{-1}(N) s^{-1} for cultivar Banata 35 (NO. 43) to 22.57 μmol(CO_2) g^{-1}(N) s^{-1} for cultivar Nancay PA (NO. 33) (Fig. 2*B*). PNUE was negatively related to N_{area} across all cultivars (Fig. 3*B*). Interestingly, PNUE was positively related to P_N across all cultivars (Fig. 4), and Nancay PA possessed both the highest P_N and PNUE among all cultivars. This suggested that PNUE and P_N can be simultaneously improved by
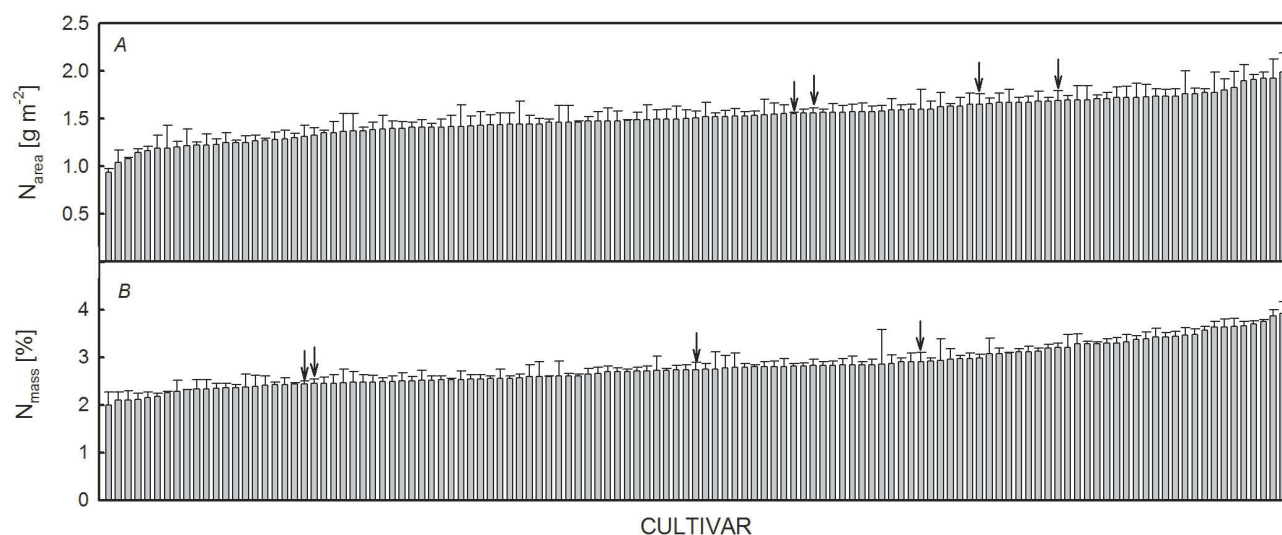
Fig. 1. Natural variations of area based (N_{area}) and mass based (N_{mass}) leaf nitrogen contents of flag leaves among 121 rice cultivars. Cultivars were ranked with the increasing value of each parameter. *Arrows* indicate the four widely grown cultivars in China, which are Huanghuazhan, Liangyoupeijiu, Shanyou 63, and Yangliangyou 6. Data were shown as means ± SD of three replications.
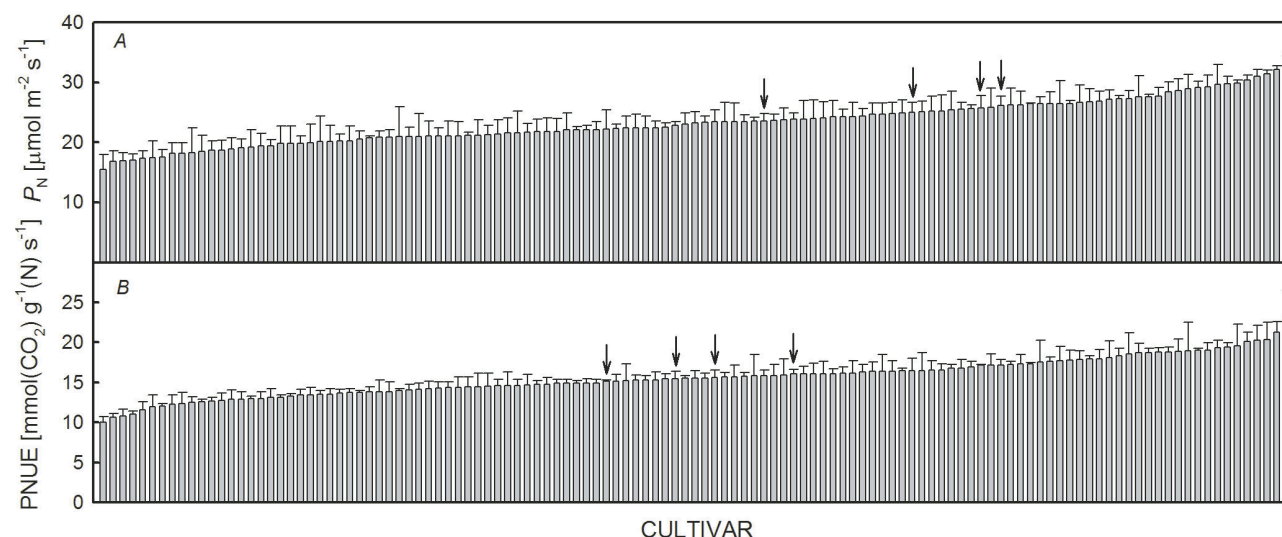

Fig. 2. Natural variations of photosynthetic rate (P_N) and photosynthetic nitrogen-use efficiency (PNUE) of flag leaves among 121 rice cultivars. Cultivars were ranked with the increasing value of each parameter. *Arrows* indicate the four widely grown cultivars in China, which are Huanghuazhan, Liangyoupeijiu, Shanyou 63, and Yangliangyou 6. Data were shown as means ± SD of four replications.

Table 1. Mean values of leaf photosynthetic rate (P_N) and its' respective area based leaf N content (N_{area}) of the cultivars in four groups. Values represent means ± SD, *different letters* indicate statistical differences at $P<0.05$.

	P_N [μmol m^{-2} s^{-1}]	N_{area} [g m^{-2}]	No. of cultivars
$P_N \geq 30$	31.5 ± 0.9[a]	1.67 ± 0.17[a]	5
$25 \leq P_N < 30$	27.0 ± 1.5[b]	1.57 ± 0.16[a]	34
$20 \leq P_N < 25$	22.4 ± 1.4[c]	1.53 ± 0.18[a]	60
$P_N < 20$	18.4 ± 1.2[d]	1.35 ± 0.22[b]	22

exploiting the natural variation of rice plants at the leaf scale. In comparison, PNUE of the widely grown cultivars 99, 107, 110, and 118 were 29.0–32.8% lower than that of the cultivar 33.

g_s and E also showed significant varietal differences between all rice cultivars. g_s ranged from 0.22 mol(H$_2$O) m^{-2} s^{-1} for Elba (NO. 60) to 0.93 mol(H$_2$O) m^{-2} s^{-1} for Belgioioso (NO. 54), while E ranged from 3.09 mmol (H$_2$O) m^{-2} s^{-1} for Ejingyou 775 (NO. 91) to 13.05 mmol (H$_2$O) m^{-2} s^{-1} for Baek Na (NO. 22). P_N and E both showed significant and positive relationships with g_s (Fig. 5). In addition, PNUE showed positive relationships with both
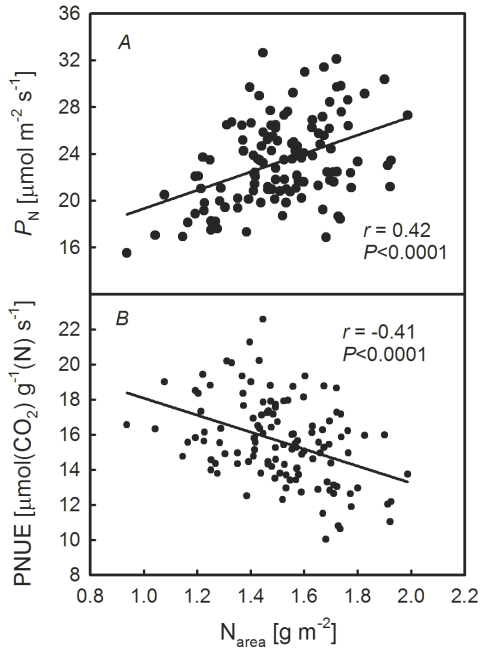
Fig. 3. Correlations of photosynthetic rate (P_N) and photosynthetic nitrogen-use efficiency (PNUE) with area based leaf nitrogen content (N_{area}) across 121 rice cultivars.
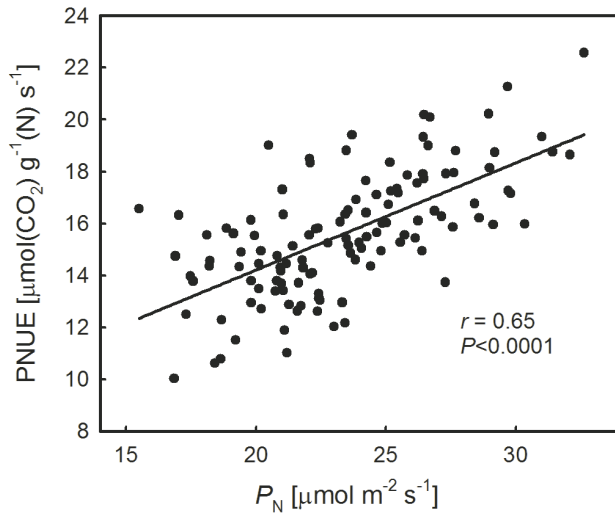

Fig. 4. Relationship between photosynthetic nitrogen-use efficiency (PNUE) and photosynthetic rate (P_N) across 121 rice cultivars.

g_s and E (Fig. 6).

LMA and P_{mass} also showed significant differences between all rice cultivars (Supplementary Table 1S). LMA ranged from 38.10 g m^{-2} for N22 (NO.108) to 73.30 g m^{-2} for Yongyou 12 (NO. 119), while P_{mass} ranged from 0.27 µmol g^{-1} s^{-1} for Yangjing 4227 (NO. 115) to 0.70 µmol g^{-1} s^{-1} for Kirmizi Celtik (NO. 68). Moreover, N_{area} was positively related to LMA, and PNUE was negatively related to LMA (Fig. 7).

N_{mass}, N_{area}, P_N, PNUE, g_s, E, LMA, and P_{mass} were all normally distributed (Fig. 1S, *supplement*). There were
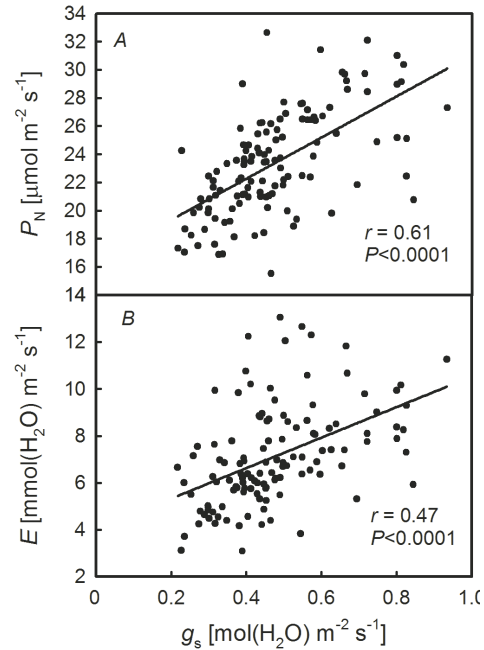

Fig. 5. Correlations of photosynthetic rate (P_N) and transpiration rate (E) with stomatal conductance (g_s) across 121 rice cultivars.

83 rice cultivars possessing N_{mass} of 2.25–3.0% (68.6% of all cultivars), while 54 rice cultivars showed N_{area} of 1.4–1.6 g m^{-2} (44.6% of all cultivars). P_N for 80 rice cultivars (accounting for 66.1% of all cultivars) ranged between 20–27 µmol m^{-2} s^{-1}, while PNUE of 86 rice cultivars ranged between 13.0–18.0 µmol g^{-1}(N) s^{-1}. There were only five cultivars whose P_N was more than 30 µmol m^{-2} s^{-1}.

Discussion

In the present study, 121 rice cultivars selected from worldwide were used to investigate the natural variation of leaf photosynthesis. PPFD of 1,500 µmol(photon) m^{-2} s^{-1} is a saturating light intensity for rice species according to previous studies (Li *et al.* 2009, Xiong *et al.* 2015, 2016, 2017), and is also a typical light intensity in midday during rice growth season at the experimental site. Therefore, this light intensity was used to study the intra-specific variation of photosynthesis in rice species.

The results showed that there was a large natural variation in leaf photosynthesis, ranging from 15.5 to 32.6 µmol m^{-2} s^{-1}. The natural variation in leaf photosynthesis would potentially be larger if more cultivars were selected, especially, when including wild species with photosynthetic rates that range from 14.3 µmol m^{-2} s^{-1} for *Oryza glumaepatula* to 37.6 µmol m^{-2} s^{-1} for *Oryza rufipogon* (Zhao *et al.* 2010).

The yield improvement of the Green Revolution has been achieved largely through improved genetics, better crop management, and protection. Many studies during the period 1970–1980 have selected cultivars with higher photosynthetic rates to improve crop yield (Moss and Musgrave 1971, Ford *et al.* 1983). In present study, P_N was not related to the year of cultivar release
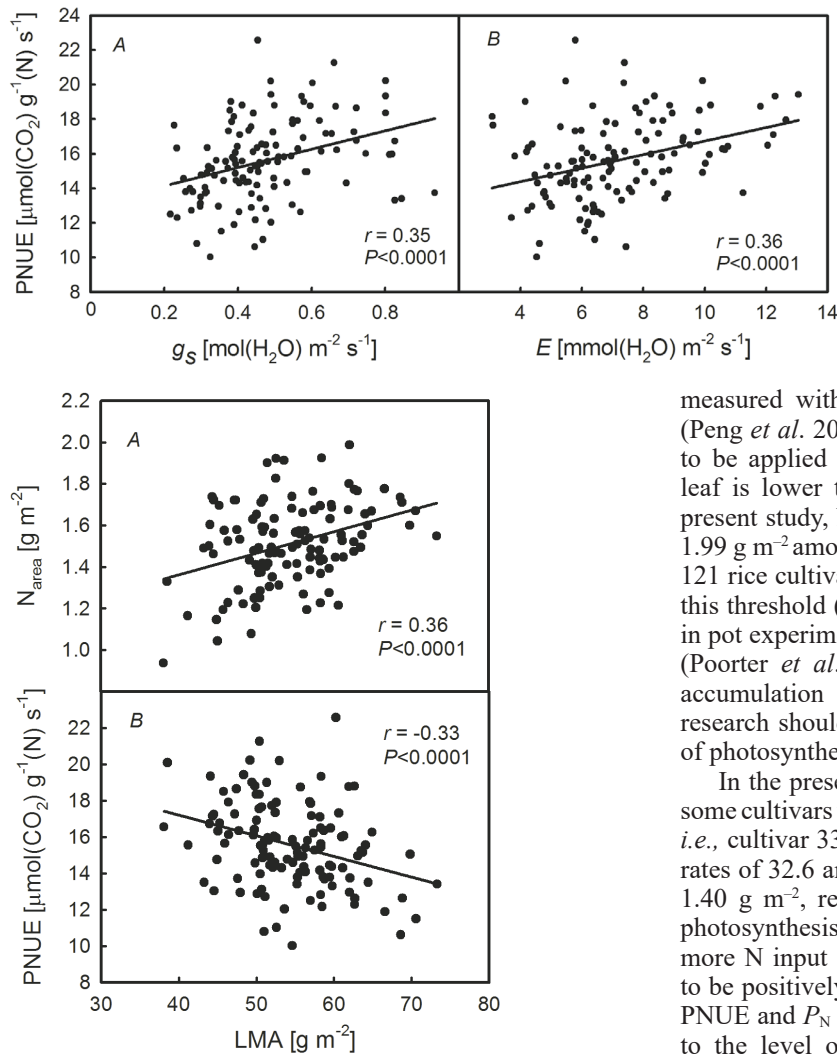
Fig. 6. Correlations of photosynthetic nitrogen-use efficiency (PNUE) with stomatal conductance (g_s) and transpiration rate (E) across 121 rice cultivars.

Fig. 7. Correlations of area based leaf nitrogen content (N_{area}) and photosynthetic nitrogen-use efficiency (PNUE) with leaf mass per area (LMA) across 121 rice cultivars.

measured with a chlorophyll meter or leaf color chart (Peng *et al.* 2010). According to this system, N is needed to be applied when N_{area} of the topmost fully expanded leaf is lower than 1.4 g m^{-2} (Peng *et al.* 1996). In the present study, N_{area} showed a large variation from 0.94 to 1.99 g m^{-2} among the tested cultivars, and N_{area} in 91 out of 121 rice cultivars (accounting for 75.2%) was higher than this threshold (Table 1S). But it should be noted that N_{area} in pot experiments is related to pot size and plant biomass (Poorter *et al.* 2012), and cultivars with high biomass accumulation are more limited by N supply. Further research should be conducted to investigate the response of photosynthesis to N supplies in these cultivars.

In the present study, N_{area} of 1.4 g m^{-2} was enough for some cultivars to achieve a high P_N of about 30 µmol m^{-2} s^{-1}, *i.e.*, cultivar 33 and 38 achieved very high photosynthetic rates of 32.6 and 29.7 µmol m^{-2} s^{-1}, with N_{area} of 1.45 and 1.40 g m^{-2}, respectively. This suggested that improving photosynthesis in rice plants did not necessarily require more N input at the leaf scale. PNUE and P_N was found to be positively related, which is an interesting finding. If PNUE and P_N of widely grown cultivars can be improved to the level of the cultivar 33 through a conventional breeding approach, we can potentially produce more rice grains with less N input.

In addition to leaf N content, CO_2 diffusion conductance is one of the limiting factors to P_N under current ambient conditions, and g_s is highly correlated with plant hydraulic status and E. In present study, P_N, E, and PNUE showed significant relationships with g_s, and PNUE was also positively related to E. This suggested that high hydraulic transport efficiency enhances the stomatal CO_2 diffusion, and finally contributes to a high PNUE. Giuliani *et al.* (2013) illustrated that there was a coordination between photosynthesis, transpiration, and structural traits in rice and wild relatives. Xiong *et al.* (2015) showed the proportion of minor vein length has a great effect on leaf hydraulic conductance (K_{leaf}) in rice. In addition, Xiong *et al.* (2017) have divided K_{leaf} into two parts – leaf hydraulic conductance inside xylem (K_x) and K_{ox} to investigate the effects of leaf morphological and anatomical traits on leaf hydraulic conductances and CO_2 diffusion conductance. And they suggested that the relationship between K_{ox} and g_m is coordinated by leaf anatomy, including the fraction of intercellular airspace (f_{ias}), mesophyll cell wall thickness, mesophyll cell wall surface area exposed to intercellular airspace per leaf area (S_m), and surface area of chloroplasts

(Fig. 2S, *supplement*). P_N of widely grown cultivars, *i.e.*, Huanghuazhan (NO. 99), Liangyoupeijiu (NO. 107), Shanyou 63 (NO. 110), and Yangliangyou 6 (NO. 118) in China, was only slightly (1.3–12.0%) higher than the mean value of all the 121 cultivars. By contrast, P_N of the cultivar 33 was 24.9–38.1% higher than these widely grown cultivars. As we know, widely grown cultivars are widely planted mainly for their high grain yield and strong resistance to stresses. It means that relying on moderate photosynthetic rate can even reach a high yield level in these widely grown cultivars. If kept their advantages in other traits and improve their P_N to 32.6 µmol m^{-2} s^{-1} (NO. 33's level) by breeding, their yield might be further increased.

Intensive crop management strategies, such as site-specific nitrogen management (SSNM) and integrated soil-crop system management, have been developed to realize high-yield crops and high-efficiency production systems. In SSNM, N application is based on leaf N status

exposed to intercellular airspace (S_c). However, there are some problems for Kx and Kox because they are difficult to be directly and accurately measured, although several approaches (including the evaporative flux method (EFM), the rehydration kinetics method (RKM), the high pressure flowmeter method (HPFM), and the vacuum pump method (VPM)) for determining K_{leaf} have been developed (Flexas *et al.* 2013, Xiong *et al.* 2017). After diffusing out of leaf veins in liquid phase, water should evaporate through existing stomata as water vapor (Rockwell *et al.* 2014). Where does the evaporation occur is unkown, although Rockwell *et al.* (2014) reported that it mainly occurred at the vascular bundles and peristomatal regions in red oak (*Quercus rubra*) trees. Moreover, environmental variations may have some impact on K_{ox}. Environmental conditions surrounding leaves can effectively influence the liquid and vapor phases in the outside-xylem regions. For example, Buckley *et al.* (2015) predicted that vapor-phase can contribute up to 39.2–44% of K_{ox} when temperature gradient between vascular plain and the epidermis is 0.2 K. It suggested that temperature can strongly affect the liquid and vapor status inside leaves. Therefore, it is necessary to develop direct and more accurate methods for K_x and K_{ox} measurements, and to study their responses to environmental changes, such as temperature and light intensity.

LMA is an important leaf structural trait, which can substantially affect leaf photosynthesis and plant growth. The dry mass of a leaf consists of a wide range of components, which can be divided into eight different classes, including structural carbohydrates, lignin, and minerals among others (Poorter *et al.* 2009). As one of the most important minerals, N content per leaf area was thus observed to be increased in high-LMA leaves (Hikosaka and Shigeno 2009), and a similar result was observed in present study (Fig. 7*A*). Moreover, PNUE was shown to be negatively related to LMA (Fig. 7*B*), which was consistent with previous studies (Takashima *et al.* 2004, Hikosaka and Shigeno 2009). This suggested that leaf anatomy can substantially affect leaf photosynthesis and PNUE. For mesophyll diffusion pathway, after diffusing through stomata, CO_2 needs to pass through the cell wall, cell membrane, cytoplasm, chloroplast envelope, and stroma to reach carboxylation sites. Thus, g_m is greatly determined by leaf anatomy, such as the fraction of intercellular air space (f_{ias}), S_m, S_c, and cell wall thickness (Evans *et al.* 2009, Scafaro *et al.* 2011, Terashima *et al.* 2011, Peguero-Pina *et al.* 2012, Tosens *et al.* 2012, Giuliani *et al.* 2013, Tomás *et al.* 2013, Muir *et al.* 2014). Generally, leaves possessing the high LMA usually have thicker cell wall, smaller intercellular air spaces (low f_{ias}, S_m, S_c) and relatively lower g_m and PNUE (Terashima *et al.* 2006, Tosens *et al.* 2012, Onoda *et al.* 2017). In addition, leaves possessing different LMA also have different N distribution. Onoda *et al.* (2017) have investigated physiological and structural tradeoffs underlying the leaf economics spectrum (LES) by analyzing a novel data compilation. It demonstrated that leaves with the high LMA, and a greater fraction of leaf dry mass in cell wall typically have a lower fraction of N in photosynthetic proteins, and thus low CO_2-diffusion rates. More research should be conducted to explore the N allocation in leaves with different LMA and its effects on PNUE.

Conclusions: The current study revealed that there are substantial natural variations in P_N, N_{area}, N_{mass}, and PNUE, and a large potential still exists to improve P_N and PNUE in widely grown rice cultivars. P_N and PNUE for four widely grown rice cultivars were 19.9–27.8% and 29.0–32.8% lower than the cultivar 33 (Nancay PA). N_{area} of 1.4 g m^{-2} was enough for some cultivars to achieve high photosynthesis of 30 μmol m^{-2} s^{-1}. PNUE showed a positive relationship with P_N across the 121 rice cultivars, and the high PNUE was associated with the high g_s, E, and low LMA.

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