Dorsoventral asymmetry of photosynthesis and photoinhibition in flag leaves of two rice cultivars that differ in nitrogen response and leaf angle

Etsushi Kumagai[a,b*], Norimitsu Hamaoka[a], Takuya Araki[c] and Osamu Ueno[a,d]

[a]Graduate School of Bioresource and Bioenvironmental Science, Kyushu University, Hakozaki 6-10-1, Higashi-ku, Fukuoka 812-8581, Japan
[b]NARO Tohoku Agricultural Research Center, Akahira 4, Shimokuriyagawa, Morioka 020-0198, Japan
[c]Faculty of Agriculture, Ehime University, Tarumi 3-5-7, Matsuyama 790-8566, Japan
[d]Faculty of Agriculture, Kyushu University, Hakozaki 6-10-1, Higashi-ku, Fukuoka 812-8581, Japan

Correspondence
*Corresponding author,
e-mail: ekumagai@affrc.go.jp

Rice is believed to show photosynthetic symmetry between adaxial and abaxial leaf sides. To verify this, we re-examined dorsoventral asymmetry in photosynthesis, chlorophyll fluorescence and anatomical traits in flag leaves of two *Oryza sativa* cultivars that differ in nitrogen (N) response and in leaf angle: 'Akenohoshi', a cultivar that can adapt to low-N (LN), with low leaf angle (more erect leaves), and 'Shirobeniya', a cultivar that is unable to adapt to LN, with higher leaf angle. Plants were grown under standard-N (SN) and LN conditions. LN leaves of both cultivars became more erect than SN, but LN Akenohoshi still had more erect ones than Shirobeniya. Contrary to results of previous studies, leaves of both cultivars showed an asymmetry in photosynthetic rate between adaxial and abaxial sides (higher on the adaxial side) under SN. SN leaves of both cultivars showed lower susceptibility to photoinhibition on the adaxial side than on the abaxial side. However, leaves of Akenohoshi showed less asymmetry in these traits under LN than under SN, whereas leaves of Shirobeniya had similar degrees of asymmetry in these traits under both SN and LN. Both cultivars also showed dorsoventral asymmetry in anatomical traits of mesophyll tissue regardless of N level, but the degree of asymmetry was lower in LN Akenohoshi. These data reveal that rice leaves exhibit dorsoventral asymmetry in photosynthetic and anatomical features, and that the degree of asymmetry varies with cultivar and N level. It is suggested that lower leaf angles (particularly in Akenohoshi) in the presence of LN represent a light acclimation to prevent photoinhibition.

Introduction

In general, leaves of C_3 dicots that show dorsoventral asymmetry develop palisade mesophyll tissue on the adaxial side and spongy mesophyll tissue on the abaxial side, and receive light mainly on the adaxial surface. The structural and functional relationships of photosynthesis in these leaves have been intensively studied (Terashima and Saeki 1983, Vogelmann 1993). Previous studies have revealed internal gradients in light intensity and photosynthetic activity within leaves. For example, the maximum photosynthetic capacity per unit mesophyll thickness is not observed in the most adaxial part of the mesophyll, whereas that expressed on chlorophyll (Chl)

Abbreviations – ASI, asymmetry index; Chl, chlorophyll; ISR, intercellular space ratio; LN, low-N; PPFD, photosynthetic photon flux density; PSII, photosystem II; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; SD, stomatal density; SL, stomatal length; SN, standard-N.

basis occurs in the palisade mesophyll cells located just below the adaxial epidermis, where the light intensity is highest (Terashima and Inoue 1985, Nishio et al. 1993, Evans and Vogelmann 2003, Terashima et al. 2009). The adaxial mesophyll tissue of C_3 dicot leaves has sun-leaf-like characteristics, while the abaxial mesophyll tissue has shade-leaf-like ones (Terashima and Evans 1988).

In contrast to the dorsoventrally asymmetrical leaves of dicots, grass species are generally thought to have symmetrical leaves. Moss (1964) compared photosynthetic rates in leaves of two C_4 grasses, maize and sugarcane. These leaves, which exhibit no differentiation of palisade and spongy mesophyll tissue layers and are amphistomatous (possess stomata on both leaf surfaces), show similar photosynthetic responses to illumination of either surface and thus are functionally symmetrical. In contrast, Soares-Cordeiro et al. (2009, 2010) recently reported that leaves of some C_4 grasses show different photosynthetic responses to adaxial and abaxial illumination. The leaves of rice, a C_3 grass, similar to those of C_4 grasses, show no obvious differentiation of palisade and spongy mesophyll tissues and are amphistomatous (Chonan 1967). Tanaka and Matsushima (1970) reported that photosynthetic rates of rice flag leaves were equal when either surface was illuminated at the same light intensity. However, more detailed studies are necessary before it can be concluded that there are no differences in the photosynthetic and anatomical characteristics between the adaxial and abaxial sides of rice leaves.

Differences in the amount of N supplied induce some differences in morphological, anatomical and physiological characteristics of rice plants. For example, N application increases the number, size and thickness of leaves (Yoshida et al. 1969). Chonan (1970) reported an increase in intercellular air space and a decrease in mesophyll cell density within rice leaves with increased N supply. Makino et al. (1983) observed a significant increase in light-saturated photosynthetic rate with increased N supply. N deficiency frequently occurs in rice cultivation. Decreases in contents of all photosynthetic components, including Chl, electron transport components and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), are usually observed in N-deficient leaves (Makino et al. 1983). In accordance with the premise that N deficiency suppresses CO_2 assimilation and leads to an increased proportion of excess excitation energy, N-deficient leaves are more susceptible to photoinhibition of photosystem II (PSII) than N-sufficient leaves (Verhoeven et al. 1997, Bungard et al. 2000, Kumagai et al. 2010).

It is well known that there are significant differences in leaf angle (degrees between panicle rachis axis and lamina measured in the proximal lamina portion) among rice cultivars (Maeda and Saka 1968). The leaf angle is also affected by nutritional conditions; N-deficient plants have a lower leaf angle (more erect leaves) than N-sufficient plants (Yoshida et al. 1969). Moreover, the upright habit has recently been introduced into new rice cultivars to increase the penetration of sunlight to lower leaves, while avoiding light saturating effects on photosynthesis in the sunlit leaves. Thus, the light environment on the adaxial and abaxial surfaces of rice leaves will depend on the cultivar and N supply. In leaves of *Silphium terebinthinaceum* (Asteraceae), which show leaf angles similar to rice leaves, changes in the light microenvironment on each leaf surface cause some changes in photosynthetic and anatomical characteristics (Poulson and DeLucia 1993). However, little information is available on the effects of N supply on these characteristics in the adaxial and abaxial sides of rice leaves.

Rice cultivars show clear differences in growth responses to N supply. In our previous studies (Kumagai et al. 2007, 2009, 2010), we investigated the effects of N deficiency on dry matter production and leaf photosynthesis in two rice cultivars, 'Shirobeniya' (an *Oryza sativa* ssp. *japonica* cultivar with a low yield) and 'Akenohoshi' (an *O. sativa* ssp. *japonica* × *indica* cross with a high yield). Under low-N (LN) conditions, the photosynthetic rate, tolerance to photoinhibition of PSII in flag leaves and dry matter production at the heading stage are all higher in Akenohoshi than in Shirobeniya. In addition, Akenohoshi has more erect flag leaves than Shirobeniya in the field.

Here, we investigated photosynthetic and anatomical dorsoventral asymmetry and the response of leaf angle in flag leaves of Akenohoshi and Shirobeniya grown under different N conditions. The aim of this study was to re-examine whether rice leaves show photosynthetic and anatomical symmetry between the adaxial and abaxial sides, as reported in previous studies, and to analyze whether the expression of any dorsoventral asymmetry is affected by differences in cultivar and N supply.

Materials and methods

Plant materials and growth conditions

Imbibed seeds of *O. sativa* ssp. *japonica* cv. Shirobeniya and subspecies *japonica* × *indica* cv. Akenohoshi were sown in nursery boxes in a glasshouse at the end of June 2008 and June 2009. At 3 weeks after sowing, the seedlings were transplanted into 8-L pots filled with sandy loam. They were divided into standard-N (SN, control) and LN groups, which were fertilized with 1.6

and 0.4 g N, respectively [as $(NH_4)_2SO_4$], as well as with 1.6 g P [as $Ca(H_2PO_4)_2$] and 1.6 g K (as KCl). Plants were grown outdoors with sufficient water throughout the experiments.

There were almost no differences in plant growth, dry matter production or photosynthetic and morphological characteristics of flag leaves between plants grown in the different years. In 2008, at 1 week after heading, eight flag leaves from four plants per cultivar grown at each N condition were selected randomly. Leaf length and width (at the greatest position) were measured, and leaf angle (degrees between panicle rachis axis and lamina measured in the proximal lamina portion; Fig. S1) was measured with a protractor as described by Maeda and Saka (1968). Thereafter, the leaves were used for measurements of photosynthesis and susceptibility to photoinhibition. After the measurements, the middle portions of four leaves from four plants were excised, frozen in N_2 and stored at $-80°C$ for determination of Chl content and Rubisco activity. The other four leaves were immediately fixed in a mixture of 5% (v/v) formaldehyde, 5% (v/v) acetic acid and 45% (v/v) ethanol in water (FAA) for anatomical observations. The aboveground parts of four plants were sampled and then dried at $80°C$ for 3 days in an oven to determine their dry weight. In 2009, at 1 week after heading, four flag leaves from four plants per cultivar grown at each N condition were selected randomly and used for assessments of the responses of Chl fluorescence parameters to light intensity.

Photosynthesis measurements

Photosynthesis was measured by using an open gas system described previously (Kumagai et al. 2007). The measurements were made on attached leaves of plants using a hand-made leaf chamber that was controlled for leaf temperature ($30.3 \pm 0.4°C$), relative humidity (RH) ($59.0 \pm 2.1\%$), ambient CO_2 ($399 \pm 13\,\mu L\,L^{-1}$) and O_2 (21%, v/v) concentrations. The leaf surface area used in the measurements was $5.9\,cm^2$ and the rate of airflow into the chamber was $16.7\,mL\,s^{-1}$. A middle portion of lamina was placed in the chamber with either the adaxial or abaxial side facing the light source. Light was provided by a metal halide lamp (LS-M180; Sumita Optical Glass Inc., Saitama, Japan) at photosynthetic photon flux density (PPFD) of 200, 500, 1000, or 1500 μmol photons $m^{-2}\,s^{-1}$. The CO_2 concentration and water vapor pressure in the reference and sample air were monitored with an infrared gas analyzer (Li-6262; LI-COR, Lincoln, NE). From the measured data, net photosynthetic rate (P_N) and stomatal conductance (g_s) were calculated as described by Long and Hallgren (1985).

Chl fluorescence measurement

Chl fluorescence was assessed by a fiber-optic portable fluorometer (PAM-2000; Walz, Effeltrich, Germany) attached to the upper window side of the leaf chamber facing the illumination source. The leaf temperature, RH and ambient CO_2 and O_2 concentrations in the chamber were maintained as described above. Lamina portions were placed in the chamber with either the adaxial or abaxial side facing the light source. Using a leaf that was dark-adapted for 30 min, we determined the minimum fluorescence (F_o) under non-photosynthetic conditions with a low-intensity measuring beam; then, we measured the maximum fluorescence (F_m) by applying a 0.8-s saturation pulse (8000 μmol photons $m^{-2}\,s^{-1}$ PPFD) to the leaf to reduce all the PSII centers. The maximal quantum yield of PSII (F_v/F_m) was calculated as ($F_m - F_o)/F_m$ (van Kooten and Snel 1990). Steady-state Chl fluorescence (F_s) under an actinic light was constantly monitored to ensure that the fluorescence had reached a plateau before a reading was taken. A 0.8-s saturation pulse was applied to determine the maximum Chl fluorescence in the light-adapted state (F_m'). The quantum yield of PSII electron transport (Φ_{PSII}), ΔpH- and xanthophyll-regulated thermal dissipation (Φ_{NPQ}), and a combined flux of fluorescence and light-independent constitutive thermal dissipation ($\Phi_{f,D}$) were calculated from the following equations proposed by Hendrickson et al. (2004): $\Phi_{PSII} = 1 - F_s/F_m'$; $\Phi_{NPQ} = F_s/F_m' - F_s/F_m$; $\Phi_{f,D} = F_s/F_m$. Measurements of these quantum yields as a function of PPFD were made in ascending order, at incident PPFD values of 100, 275, 580, 860, 1290, and 1900 μmol photons $m^{-2}\,s^{-1}$ at the leaf surface.

Photoinhibitory treatment

Flag leaves were set in the leaf chamber as described above and either side was exposed for 2 h to a high PPFD of 2000 μmol photons $m^{-2}\,s^{-1}$ provided by the metal halide lamp. Before and after exposure to the high PPFD, the leaves were dark-adapted for 30 min, and F_v/F_m of the illuminated leaf side was measured as described above, known as F_v/F_{mM} before exposure and F_v/F_{mPI} after exposure.

Determination of Chl content and Rubisco activity

Three excised disks (total area $= 2.36\,cm^2$) per leaf were soaked in 96% (v/v) ethanol in the dark for 2 days until the color was leached. Then, the Chl content in the ethanol solution was measured spectrophotometrically according to Wintermans and de Mots (1965).

A total of 10 excised disks (total area $= 7.86\,cm^2$) per leaf were ground with a pestle in a mortar (on ice)

containing sea sand, 25 mg polyvinylpyrrolidone and 1 mL of grinding medium, consisting of 50 mM HEPES-KOH (pH 7.5), 0.2 mM EDTA, 2.5 mM MgCl$_2$, 5 mM dithiothreitol and 0.7% (w/v) bovine serum albumin. Homogenates were centrifuged at 10 000 g for 5 min at 4°C. The supernatants were used for assays of Rubisco activity. For the assay, the supernatant was pre-incubated for 10 min in the presence of 10 mM NaHCO$_3$ and 10 mM MgCl$_2$ at 25°C to obtain maximum activation. Rubisco activity was measured spectrophotometrically in 1-ml reaction mixture at 25°C according to Ueno and Sentoku (2006).

Measurement of stomatal density and stomatal length

The FAA-fixed leaf blades were incubated in 70% (v/v) ethanol at 80°C for 12 h four times. Thereafter, they were transferred to 80% (v/v) lactic acid and incubated at 80°C for 12 h twice, and finally stored in chloral hydrate-saturated ethanol. The stomata on each epidermis were observed under a light microscope (Biophot; Nikon, Tokyo, Japan). The stomatal density (SD) was determined in a field of 0.322 mm^2 at 400× magnification with five replications for each surface of each leaf sample. The stomatal length (SL) of 20 stomata selected randomly on a photomicrograph (800× magnification) was measured with an ocular micrometer with five replications for each surface of each leaf sample.

Quantification of intercellular space in mesophyll tissue

The FAA-fixed leaf blades were cut into segments approximately 1 mm wide with a razor blade, and the segments were then washed several times with distilled water. Thereafter, they were immersed in 25% (w/v) hydrofluoric acid for 24 h to remove silica within the tissues. After washing, the segments were fixed for 1.5 h in 3% (w/v) glutaraldehyde in 50 mM NaH$_2$PO$_4$ buffer (pH 6.8) at room temperature. After washing with NaH$_2$PO$_4$ buffer, the segments were post-fixed for 2 h in 2% (w/v) OsO$_4$ in NaH$_2$PO$_4$ buffer and then dehydrated at room temperature through a graded acetone series The segments were embedded in epoxy resin (Quetol-651; Nisshin-EM, Tokyo, Japan) and polymerized at 70°C for 24 h. Mesophyll cells of rice leaves exhibit a complex structure with lobes in transverse view, which makes quantification of the anatomical features difficult, but the cells appear as regularly stacked files lying vertically with respect to epidermis cells in longitudinal view (Chonan 1967, 1970, Sage and Sage 2009). Thus, the intercellular space ratio (ISR) of each leaf side was

assessed in longitudinal sections. Semi-thin sections of leaves (approximately 1 μm thick) were longitudinally cut with glass knives, and were then stained with toluidine blue O on glass slides.

Photomicrographs of the sections were taken at a magnification of 800× with five replications for each leaf sample. A mesophyll field of 900 μm^2 (30 × 30 μm) beneath the epidermis of each side was randomly selected in the photomicrograph. The area of intercellular space was measured by using image analysis software (IMAGE J 1.40g; NIH, Bethesda, MD), and then the ISR was calculated as the ratio of the intercellular space area to the total mesophyll sectional area.

Asymmetry indices

The asymmetry indices (ASIs) for gas exchange, Chl fluorescence and anatomical parameters were calculated as the ratio of the value of the abaxial side to that of the adaxial side. By definition, if a given parameter shows the same feature in both sides, its ASI is 1.00.

Statistical analysis

Differences of mean values in the plant aboveground dry weight and the length, width, angle, thickness, Chl content and Rubisco activity of leaves were assessed with Tukey's multiple comparison test. Gas exchange, Chl fluorescence and anatomical parameters of the adaxial and abaxial sides of leaves were likewise analyzed. All statistical tests were performed using SIGMASTAT V. 3.1 software (Systat Software Inc., Chicago, IL).

Results

Morphological and physiological characteristics of flag leaves

Relative to SN, LN resulted in a significant decrease in the plant aboveground dry weight of both cultivars, but more in Shirobeniya (Table 1). Plants grown under LN had smaller and thinner leaves than those grown under SN. Under both conditions, Akenohoshi had significantly greater and thicker flag leaves than Shirobeniya. LN also decreased the leaf angle of both cultivars, i.e. flag leaves were more erect than under SN. Furthermore, the leaf angle of Akenohoshi was significantly lower than that of Shirobeniya under both SN and LN. Thus, the LN Akenohoshi plants had the most erect flag leaves. LN decreased the Chl content and Rubisco activity in both cultivars. Under LN, Rubisco activity was significantly higher in Akenohoshi than in Shirobeniya, although there was no difference in Chl content between the two cultivars.

Table 1. Aboveground plant dry weight at maturity, morphological features, Chl content and Rubisco activity of flag leaves at the heading stage of two rice cultivars grown under SN or LN. Data are mean $\pm$ SE of four plants per cultivar in each N condition. Values followed by the same letters were not significantly different ($P < 0.05$) by Tukey's test. Values in parentheses are the ratios of the LN and SN values of each cultivar.

Treatment	Cultivar	Aboveground plant dry weight (g plant^{-1})	Leaf length (cm)	Leaf width (cm)	Leaf angle (°)	Leaf thickness (µm)	Chl content (g m^{-2})	Rubisco activity (µmol m^{-2} h^{-1})
SN	Akenohoshi	101.5 $\pm$ 1.7a	45.6 $\pm$ 0.9a	1.80 $\pm$ 0.03a	14.3 $\pm$ 1.1b	120.3 $\pm$ 6.0a	0.55 $\pm$ 0.01a	178.3 $\pm$ 4.2a
	Shirobeniya	98.3 $\pm$ 1.2a	31.5 $\pm$ 0.9b	1.50 $\pm$ 0.03b	22.6 $\pm$ 1.0a	94.5 $\pm$ 3.6c	0.63 $\pm$ 0.01a	170.8 $\pm$ 2.2a
LN	Akenohoshi	41.4 $\pm$ 1.3b (0.41)	33.2 $\pm$ 1.2b (0.73)	1.37 $\pm$ 0.03c (0.76)	6.0 $\pm$ 0.9c (0.42)	100.0 $\pm$ 4.2b (0.83)	0.38 $\pm$ 0.01b (0.69)	96.9 $\pm$ 3.0b (0.54)
	Shirobeniya	30.8 $\pm$ 1.7c (0.31)	22.3 $\pm$ 1.0c (0.71)	1.08 $\pm$ 0.04d (0.72)	15.0 $\pm$ 1.1b (0.66)	90.0 $\pm$ 3.4c (0.95)	0.41 $\pm$ 0.01b (0.65)	75.2 $\pm$ 4.2c (0.44)
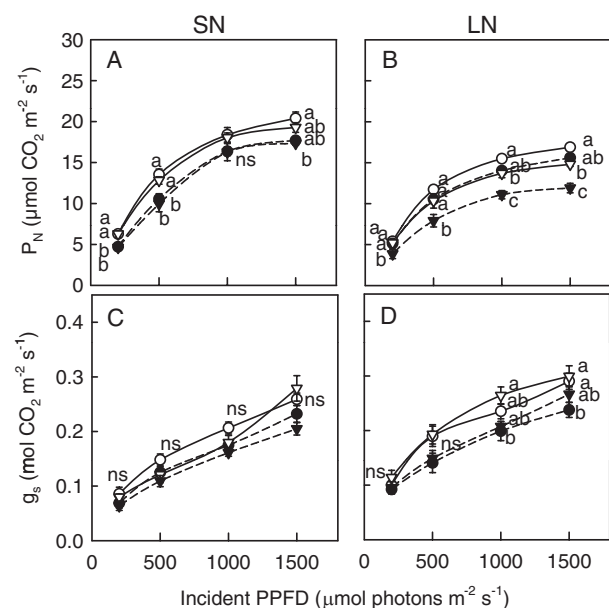
Fig. 1. Light-response curves for net photosynthetic rate (P_N; A, B) and stomatal conductance (g_s; C, D) of flag leaves when each surface was illuminated. Cultivars Akenohoshi and Shirobeniya were grown under SN (A, C) or LN (B, D). Data are mean $\pm$ SE of four plants per cultivar in each N condition. Values followed by the same letters within each PPFD were not significantly different ($P < 0.05$) by Tukey's test. ns, no significant difference among all values; circles, Akenohoshi; triangles, Shirobeniya; open symbols, adaxial illumination; closed symbols, abaxial illumination.

P_N and g_s under illumination of opposite leaf sides

As PPFD increased, P_N and g_s increased proportionally regardless of the differences in cultivar, N level and the side of the leaf illuminated (Fig. 1). When illuminated on either surface, the P_N of flag leaves was lower in plants grown under LN (Fig. 1A, B). In leaves of plants grown under SN, the P_N of the two cultivars did not differ at any PPFD with illumination of the same surface (Fig. 1A). In leaves of plants grown under LN, however, P_N at high PPFD values (1000 and 1500 µmol photons m^{-2} s^{-1})

was significantly higher in leaves of Akenohoshi than in those of Shirobeniya (Fig. 1B). In leaves of plants grown under SN, at low and middle PPFD values (200 and 500 µmol photons m^{-2} s^{-1}), P_N was higher with adaxial illumination than with abaxial illumination, whereas at higher PPFD values there was no clear difference in P_N between adaxial and abaxial illumination in either cultivar (Fig. 1A). In plants grown under LN, the P_N of Akenohoshi leaves did not differ significantly between adaxial and abaxial illumination over a wide range of PPFD values; in contrast, the P_N of Shirobeniya leaves was significantly higher with adaxial illumination. As a result, the ASI of P_N in leaves of plants grown under LN ranged from 0.90 to 0.95 in Akenohoshi and from 0.74 to 0.81 in Shirobeniya (Table 2).

In leaves of plants grown under either N treatment, there were no clear differences in g_s in either cultivar between abaxial and adaxial illumination (Fig. 1C, D), except that at a PPFD of 1500 µmol photons m^{-2} s^{-1}, the g_s of LN Akenohoshi leaves was lower with abaxial illumination than with adaxial illumination (Fig. 1D).

Photochemical properties under illumination of different leaf sides

There was almost no difference in F_v/F_{mM} between SN and LN (Fig. 2A, B), whereas F_v/F_{mPI} was lower under LN (Fig. 2C, D). Under SN, F_v/F_{mM} values were similar regardless of cultivar or the leaf side illuminated (Fig. 2A), whereas under LN, Akenohoshi had significantly higher F_v/F_{mM} values than Shirobeniya with either leaf side illuminated (Fig. 2B). F_v/F_{mPI} was less than F_v/F_{mM} regardless of cultivar, N level or the leaf side illuminated. Under the same N status, F_v/F_{mPI} was significantly different between cultivars when the same leaf side was compared. The difference was greater under illumination of the abaxial side (Fig. 2C, D). Under SN, F_v/F_{mPI} values in both cultivars were significantly higher with illumination of the adaxial surface. Under LN, however, there was no significant difference in F_v/F_{mPI} of

Table 2. ASIs for net photosynthetic rate (P_N), quantum yields of PSII electron transport (Φ_{PSII}) and light-dependent thermal dissipation (Φ_{NPQ}) of flag leaves of cultivars Akenohoshi and Shirobeniya grown under SN and LN

		SN		LN	
Trait	PPFD (μmol photons m^{-2} s^{-1})	Akenohoshi	Shirobeniya	Akenohoshi	Shirobeniya
P_N	200	0.75	0.74	0.95	0.74
	500	0.78	0.78	0.90	0.80
	1000	0.89	0.90	0.91	0.81
	1500	0.87	0.90	0.92	0.80
Φ_{PSII}	100	1.01	1.01	0.99	0.98
	275	1.01	0.98	0.99	0.91
	580	0.97	0.92	1.01	0.92
	860	0.89	0.90	1.00	0.85
	1290	0.87	0.84	0.97	0.77
	1900	0.83	0.75	0.96	0.72
Φ_{NPQ}	100	1.07	1.18	0.90	1.23
	275	1.07	1.28	0.93	1.33
	580	1.20	1.36	0.90	1.17
	860	1.31	1.26	0.97	1.19
	1290	1.24	1.23	1.00	1.19
	1900	1.16	1.19	1.00	1.15
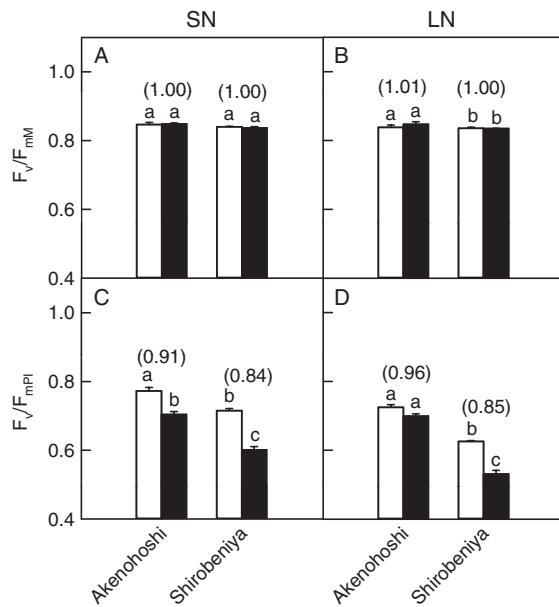
Fig. 2. Maximal quantum yield of PSII (F_v/F_m) of adaxial (open bars) and abaxial (closed bars) surfaces of flag leaves of cultivars Akenohoshi and Shirobeniya before and after exposure to high PPFD illumination for 2 h. Plants were grown under SN (A, C) or LN (B, D). F_v/F_{mM} measured before high illumination (A, B) and F_v/F_{mPI} are F_v/F_m measured after high illumination (C, D). Data are mean ± SE of four plants per cultivar for each N condition. Values in parentheses are the ratios of abaxial to adaxial values (ASI). Bars under the same letters in each N condition were not significantly different ($P < 0.05$) by Tukey's test.

Akenohoshi between adaxial and abaxial illumination. As a result, the ASI of F_v/F_{mPI} for Akenohoshi grown under LN was near 1.00, but that for Shirobeniya was lower at 0.85.

As PPFD increased, Φ_{PSII} decreased in a similar manner regardless of cultivar, N level and leaf side illuminated (Fig. 3A, B). Under SN, however, at middle and high PPFD values (860, 1290 and 1900 μmol photons m^{-2} s^{-1}), Φ_{PSII} tended to be higher with adaxial illumination in both cultivars (Fig. 3A). Under LN, in leaves of Shirobeniya, Φ_{PSII} was significantly higher with adaxial illumination, whereas in leaves of Akenohoshi, there were no significant differences in Φ_{PSII} between adaxial and abaxial illumination over a wide range of PPFD (Fig. 3B). As a result, Akenohoshi showed higher values of ASI (0.96–1.01) of Φ_{PSII} than Shirobeniya (Table 2).

At increasing PPFD, Φ_{NPQ} increased in a similar manner in leaves of both cultivars with illumination of either side (Fig. 3C, D). Under SN, Φ_{NPQ} tended to be higher with abaxial illumination in both cultivars. Under LN, however, in leaves of Akenohoshi, Φ_{NPQ} values were very similar with illumination of either leaf side, but in leaves of Shirobeniya, Φ_{NPQ} was higher with abaxial illumination. As a result, ASI values of Φ_{NPQ} for Shirobeniya were higher than those for Akenohoshi (Table 2).

$\Phi_{f,D}$ values were almost constant as PPFD increased in both cultivars and with either leaf side illuminated (Fig. 3E, F), and there was no significant difference in $\Phi_{f,D}$ under illumination of either leaf side in either cultivar at any PPFD.

Stomatal characteristics and ISR

SD was greater and SL was somewhat smaller under LN (Fig. 4A–D). In both cultivars, SD was higher on the abaxial surface (Fig. 4A, B). However, the ASI of the
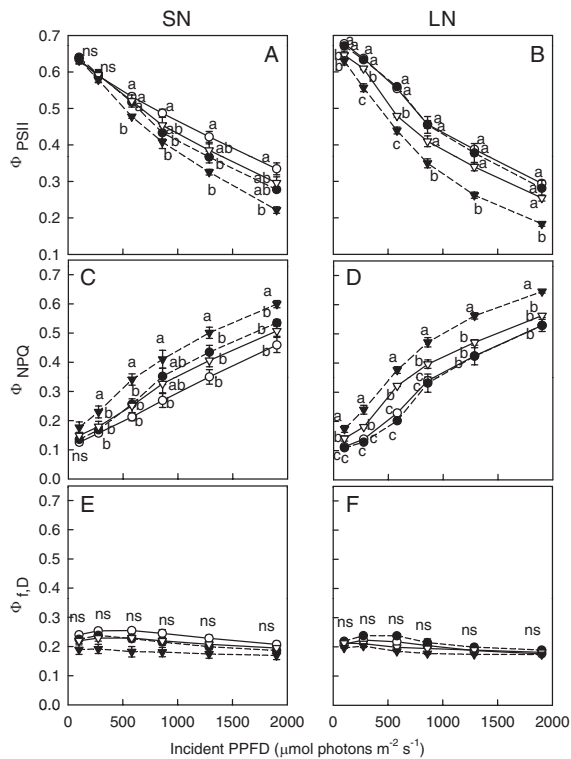
Fig. 3. Light-response curves for quantum yields of PSII electron transport (Φ_{PSII}; A, B), light-dependent thermal dissipation (Φ_{NPQ}; C, D) and fluorescence and light-independent constitutive thermal dissipation ($\Phi_{f,D}$; E, F) of both surfaces of flag leaves of cultivars Akenohoshi and Shirobeniya. Plants were grown under SN (A, C, E) or LN (B, D, F). Data are mean $\pm$ SE of four plants per cultivar in each N condition. Values labeled with the same letters within each PPFD were not significantly different ($P < 0.05$) by Tukey's test. ns, no significant difference among all values; circles, Akenohoshi; triangles, Shirobeniya; open symbols, adaxial illumination; closed symbols, abaxial illumination.

Fig. 4. SD (A, B), SL (C, D) and ISR (E, F) of adaxial (opened bars) and abaxial (closed bars) sides of flag leaves of cultivars Akenohoshi and Shirobeniya. Plants were grown under SN (A, C) or LN (B, D). Data are mean $\pm$ SE of values from five digital images of leaf samples per plant from four plants per cultivar in each N condition. Values in parentheses are the ratios of abaxial to adaxial values (ASI). Bars under the same letters in each N condition were not significantly different ($P < 0.05$) by Tukey's test.
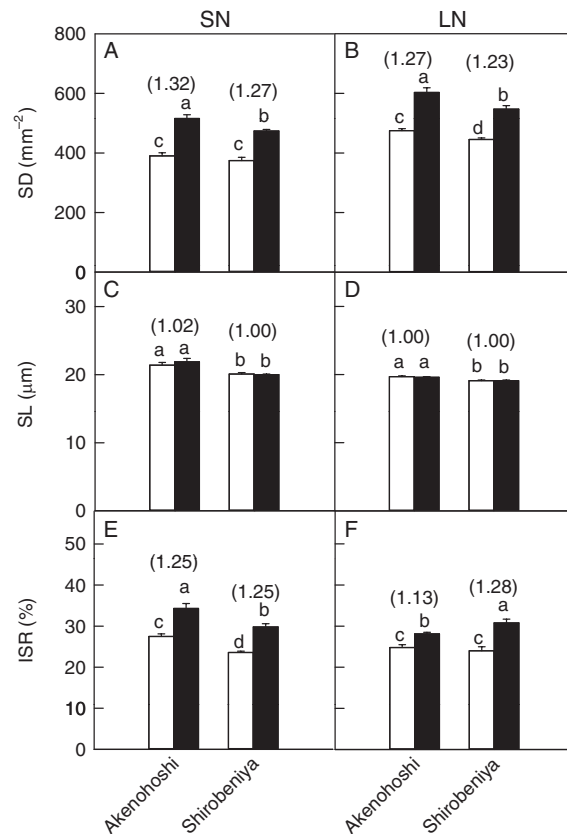
SD of LN leaves was slightly lower than that of the SD of SN leaves. Under LN, the SD of both leaf surfaces was significantly higher in Akenohoshi. There was no significant difference in SL between the leaf surfaces of either cultivar (Fig. 4C, D). Under both N treatments, however, Akenohoshi had somewhat more stomata on each leaf side than Shirobeniya.

ISR was lower on the adaxial side under each N treatment (Fig. 4E, F). In Akenohoshi, but not Shirobeniya, the ISR of both leaf sides was lower under LN, and the degree of decrease was greater on the abaxial side. As a result, the ASI of ISR of Akenohoshi was lower under LN.

Discussion

Overall responses of leaves to LN supply

N deficiency reduced dry matter production, photosynthetic capacity and tolerance of photoinhibition in the flag leaves of the two rice cultivars, especially in Shirobeniya (Table 1, Figs 1 and 2). These results are in good agreement with those of our previous studies (Kumagai et al. 2007, 2009, 2010). N deficiency resulted in a decline in the angle of flag leaves in both cultivars (Table 1), which is consistent with the results of Yoshida et al. (1969). Furthermore, under LN, flag leaves of Akenohoshi were more erect than those of Shirobeniya. Thus, the adaxial and abaxial surfaces of flag leaves in the LN plants of Akenohoshi would be more equally irradiated during the day than those of SN plants, as discussed in latter section.

Dorsoventral asymmetry in CO$_2$ exchange characteristics

We determined the responses of P$_N$ to illumination on each side of the flag leaf. It should be noted that

the determined values of P_N included data from both leaf sides. However, when illuminating one side of the lamina, because the transmittance of rice leaves is about 10% (Agarie et al. 1996), the light transmitted to the opposite leaf side would be negligible in driving photosynthesis (especially at low incident PPFD). Under SN, two cultivars showed lower values of ASI at low and middle PPFD values (Table 2). Under LN, however, they showed different ASI values of P_N; ASI of Akenohoshi was approached to 1.00, whereas that of Shirobeniya was still not changed. These results demonstrate that rice leaves have a dorsoventral asymmetry of P_N that depends on N level and cultivar. These results disagree with those of Tanaka and Matsushima (1970), who reported no difference in the light response curves of P_N between adaxial and abaxial illumination of flag leaves. This discrepancy might be caused by differences in the cultivars used and the level of N fertilizer administered. Soares-Cordeiro et al. (2009) have also reported that P_N of leaves of the C_4 grasses *Paspalum dilatatum* and *Zea mays* is higher with adaxial illumination, whereas that of the C_3 grass *Triticum turgidum* is the same with illumination of either leaf side.

Dorsoventral asymmetry in anatomical leaf characteristics

The patterns of stomatal size and density found in this study correspond with the results of a previous study of rice cultivars (Kawamitsu et al. 1996). LN resulted in slightly lower SL than SN, but the ASI values of SL were equal. In species of the *Compositae*, increased light intensity promoted an increase in the SD of the adaxial surface (a change toward amphistomaty) (Mott and Michaelson 1991). In our study, SD was higher under LN, as shown in the study of Yoshida and Ono (1978), but SD was higher on the abaxial surface regardless of N status.

The density of mesophyll cells influences P_N; denser mesophyll cells may accumulate a higher content, per unit leaf area, of photosynthetic enzymes such as Rubisco (Evans and Loreto 2000) that may lead to a higher P_N. Irrespective of N level and cultivar, the ISR was higher on the abaxial side (Fig. 4E, F), suggesting that rice leaves have denser mesophyll on the adaxial side. Our data thus reveal that rice leaves have a dorsoventral asymmetry in their mesophyll structure, contrary to previous report (Chonan 1967). It is interesting to note that the ASI of the ISR for Akenohoshi approached 1.0 under LN (Fig. 4F), which would suggest a symmetrical distribution of chloroplasts and Rubisco between the leaf sides. In contrast, the ASI of the ISR for Shirobeniya was 1.28 under LN (Fig. 4F), which results from sparser mesophyll cells and probably lower accumulation of

chloroplasts and Rubisco on the abaxial side. This ISR asymmetry may partially explain why LN leaves of Shirobeniya had a lower P_N under abaxial illumination.

## Dorsoventral asymmetry in Chl fluorescence parameters

When Chl fluorescence is measured with a conventional PAM fluorometer, it represents a mixture of signals emitted from the mesophyll cell layers facing the detector, because the incident light (blue and/or red) cannot penetrate deeply within the leaf (Bornman et al. 1991). Using this system, we first provided the data of determined Chl fluorescence in the adaxial and abaxial sides of rice leaves. Our data demonstrate that leaves of rice grown under SN have dorsoventral asymmetry in their susceptibility to photoinhibition (Fig. 2C). Previous studies have also found that the fluorescence characteristics generally differ between the two leaf sides (Schreiber et al. 1977); for example, in leaves of sunflower (Evans et al. 1993) and spinach (Sun et al. 1996), the adaxial side is more tolerant (less reduction of F_v/F_m) to high light intensities than the abaxial side.

Our study revealed that the two cultivars have different leaf side-specific photochemical traits, which respond differently to LN. In Akenohoshi under LN, the differences in F_v/F_{mPI}, Φ_{PSII} and Φ_{NPQ} between the leaf sides were smaller than under SN, approaching an ASI of 1.0 (Fig. 2D, Table 2). In contrast, in Shirobeniya under LN, the differences remained as large as those under SN. As discussed in the next section, this cultivar-based difference is associated with the different responses of leaf angle in the two cultivars to N status. In addition, the mesophyll structure of the two cultivars responded differently to the N status during growth. In only Akenohoshi under LN, the leaves had a nearly symmetrical mesophyll structure (Fig. 4). Takahashi et al. (1994) showed that Chl fluorescence emission in rice leaves reaches a maximum at around 20 µm depth from the adaxial leaf surface. Thus, the Chl fluorescence data in our study represent the signal from the mesophyll beneath the epidermis of the illuminated side, where the ISR was evaluated. The greater symmetry of PSII electron transport activity and susceptibility to photoinhibition in Akenohoshi under LN than under SN could be at least partially accounted for by the more symmetrical mesophyll structure under LN.

Ecophysiological considerations of dorsoventral asymmetry of rice leaves

Our study indicates that the anatomical and photosynthetic dorsoventral asymmetry in rice leaves is modulated by the interaction of N level and genetic factors
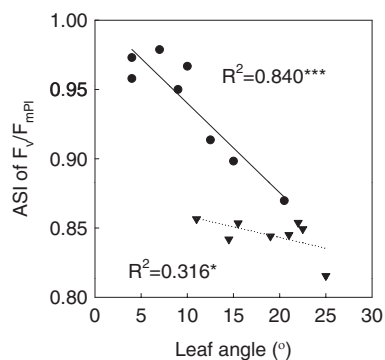
Fig. 5. Correlations between leaf angle and the ASI of F_V/F_{mPI} in Akenohoshi (closed circles) and Shirobeniya (closed triangles). $*P < 0.05$ and $***P < 0.001$.

(cultivar differences). The N level causes a cultivar-specific response of leaf angle, which seems to affect the development of dorsoventral asymmetry in the leaves. When Akenohoshi was grown under LN, it developed even more erect leaves with symmetrical anatomical and photosynthetic traits. In contrast, Shirobeniya developed weakly erect leaves with asymmetrical traits. These observations show that rice leaves can modulate the extent of dorsoventral asymmetry, via leaf angle, in their response to an external factor (light) and N availability, and some genetic factors are involved in this capability. The leaf angle largely affects the amount of solar radiation that the leaf surface receives and its dilution on the intercepting lamina surface. Indeed, our preliminary experiments confirmed that the integrated amount of solar radiation on the adaxial and abaxial surfaces of flag leaves in the two rice cultivars was strongly influenced by the leaf angle, and that flag leaves of Akenohoshi grown under LN received almost the same amount of solar radiation on each surface (Fig. S2). Therefore, we propose that the change of light environment in leaves caused by the decreased leaf angle induces the development of more symmetrical leaves in Akenohoshi under LN. Previous studies have confirmed that horizontal and dorsoventral leaves of some dicots, such as sunflower (Evans et al. 1993) and *Chenopodium album* (Oguchi et al. 2003), can alter their mesophyll and chloroplast structure in response to a change of light environment. Our study suggests that rice plants also have this ability to acclimatize the anatomical and photosynthetic traits of leaves in response to differential leaf-side illumination. Both Akenohoshi and Shirobeniya showed negative relationships between leaf angle and the ASI of F_V/F_{mPI} (Fig. 5). These data suggest that the leaf angle determines the degree of dorsoventral asymmetry in the susceptibility to photoinhibition of PSII.

The flag leaves of Akenohoshi became more erect under LN than under SN, resulting in higher tolerance of photoinhibition on both leaf sides than in those of Shirobeniya. The adaxial surfaces of leaves in a horizontal position receive a greater daily total solar radiation than the abaxial surfaces (Poulson and DeLucia 1993). The advantage of upright leaves in rice has been clarified by the observation that the extent of photoinhibition increases when leaves are forced into a horizontal position (Murchie et al. 1999, Chen et al. 2003). The degree of photoinhibition of PSII is also correlated with the period of exposure to light (Werner et al. 2001). Therefore, it seems that Akenohoshi mitigates photoinhibition by weakening incident solar radiation through the decrease in leaf angle, which may result in the maintenance of P_N under LN. In rice, the upright posture of leaves promotes the penetration of solar radiation to lower leaves and helps to optimize the light distribution in the canopy (Murchie et al. 1999). Under unfavorable conditions such as N deficiency, photoinhibition inhibits photosynthesis and probably also dry matter production (Kumagai et al. 2009). We suggest that the responses of leaves of Akenohoshi to N deficiency contribute to minimizing the risk of photoinhibition on both leaf sides while maximizing the daily carbon gain in the canopy.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure. S1. The leaf angle (degrees between panicle rachis axis and lamina measured in the proximal lamina portion) of rice plants defined in this study.

Figure. S2. Integrated solar radiation on the adaxial and abaxial surfaces of flag leaves of cultivars Akenohoshi and Shirobeniya during 10 days after heading.

Edited by T. Vogelmann