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RESEARCH PAPER

Identification and characterization of genomic regions on chromosomes 4 and 8 that control the rate of photosynthesis in rice leaves

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Abstract

DNA marker-assisted selection appears to be a promising strategy for improving rates of leaf photosynthesis in rice. The rate of leaf photosynthesis was significantly higher in a high-yielding *indica* variety, Habataki, than in the most popular Japanese variety, Koshihikari, at the full heading stage as a result of the higher level of leaf nitrogen at the same rate of application of nitrogen and the higher stomatal conductance even when the respective levels of leaf nitrogen were the same. The higher leaf nitrogen content of Habataki was caused by the greater accumulation of nitrogen by plants. The higher stomatal conductance of Habataki was caused by the higher hydraulic conductance. Using progeny populations and selected lines derived from a cross between Koshihikari and Habataki, it was possible to identify the genomic regions responsible for the rate of photosynthesis within a 2.1 Mb region between RM17459 and RM17552 and within a 1.2 Mb region between RM6999 and RM22529 on the long arm of chromosome 4 and on the short arm of chromosome 8, respectively. The designated region on chromosome 4 of Habataki was responsible for both the increase in the nitrogen content of leaves and hydraulic conductance in the plant by increasing the root surface area. The designated region on chromosome 8 of Habataki was responsible for the increase in hydraulic conductance by increasing the root hydraulic conductivity. The results suggest that it may be possible to improve photosynthesis in rice leaves by marker-assisted selection that focuses on these regions of chromosomes 4 and 8.

Key words: Hydraulic conductance, hydraulic conductivity, leaf water potential, nitrogen content, photosynthetic rate, quantitative trait locus, rice, root surface area, stomatal conductance, water stress.

Introduction

Increases in rates of leaf photosynthesis are important if the yield potential of rice (*Oryza sativa* L.) is to be increased since the rates of photosynthesis of the individual leaves affect dry matter production via photosynthesis within the canopy (Long *et al.*, 2006; Murchie *et al.*, 2009). The rate of single-leaf photosynthesis can be categorized in terms of three parameters. One is the rate of photosynthesis, which is measured at full leaf expansion under saturating light, the ambient atmospheric concentration of CO_2, an optimum temperature, and a low vapour pressure deficit, and it will be referred to here as the rate of maximum photosynthesis (Murata, 1961; Makino *et al.*, 1988). A second parameter is the extent of the midday and afternoon depression of the rate of photosynthesis that results from abiotic stresses, such as water stress (Hirasawa and Ishihara, 1992; Ishihara, 1995; Hirasawa and Hsiao, 1999). The third parameter is the reduction in the rate of photosynthesis that accompanies senescence (Makino *et al.*, 1984; Jiang *et al.*, 1999).

The present study focused on the rate of maximum photosynthesis. The activity of Rubisco limits photosynthesis at lower intercellular concentrations of CO_2 (C_i; Farquhar *et al.*, 1980; Makino *et al.*, 1985). As C_i increases, the electron transport capacity of the photosynthetic machinery limits photosynthesis (Farquhar *et al.*, 1980). In addition, at higher values of C_i, the availability of inorganic phosphate also contributes to the limitation of photosynthesis (Sharkey, 1985). In rice leaves, the photosynthesis is limited by Rubisco content at ambient atmospheric concentration of CO_2 (Makino *et al.*, 1985). In turn, the Rubisco content of each leaf is closely correlated with the leaf nitrogen content (Makino *et al.*, 1984; Evans, 1989).

There are several reports of comparisons of rates of photosynthesis in individual leaves (Ishii, 1995), among varieties of *japonica* rice (Murata, 1961; Sasaki and Ishii, 1992; Ishii, 1995; Osada, 1995), and among varieties including *indica* and *japonica* rice and other species of *Oryza* (Takano and Tsunoda, 1971; Cook and Evans, 1983; Yeo *et al.*, 1994; Osada, 1995; Xu *et al.*, 1997; Masumoto *et al.*, 2004). Some significant differences among varieties, within species of *Oryza*, and among progeny plants derived from crosses between species were described. Plant breeding that was directed against lodging has enhanced rates of leaf photosynthesis by increasing the nitrogen content of leaves under conditions of heavy nitrogen application (Osada, 1995). On the other hand, Makino *et al.* (1987) reported that differences in the Michaelis constant (CO_2) and maximum initial velocity per milligram of protein in the carboxylase reaction of Rubisco and in the ratio of Rubisco protein to total soluble protein were very small among cultivars of *japonica*, *indica*, *indica*×*japonica*, and *javanica* types. It was also reported that there was barely any difference in terms of the relationship between the nitrogen and the Rubisco content of leaves among several *japonica* and *indica* varieties (Hirasawa *et al.*, 2010).

Varietal differences in stomatal conductance, which regulates the supply of CO_2 from the air to the interior of the leaf, have often been observed (Maruyama and Tajima, 1990; Ohsumi *et al.*, 2007) and, thus, stomatal conductance might be an important factor in the varietal differences among rates of photosynthesis (Ohsumi *et al.*, 2007). However, since stomatal conductance is influenced not only by hydraulic conductance (Hirasawa *et al.*, 1992*b*; Holbrook, 2006; Hopkins and Huner, 2008) but also by leaf nitrogen content (Ishihara, 1995; Makino *et al.*, 1988), it is important to analyse the effects of nitrogen on these varietal differences in rates of photosynthesis; however, little attention has been paid to these effects to date. It was demonstrated previously that the rate of photosynthesis was higher in the *indica* variety Takanari at the same rate of nitrogen application than in *japonica* varieties, but the rate in Takanari was also higher even when levels of leaf nitrogen were the same, as a result of the higher stomatal conductance of Takanari (Hirasawa *et al.*, 2010).

Since the measurements of photosynthesis have been time consuming and labour intensive, leaf photosynthesis has not been considered as a selection objective in plant breeding. Various DNA markers have been developed in rice, whose entire genome has been sequenced (Sasaki, 2003; International Rice Genome Sequencing Project, 2005), and new mapping populations, such as chromosome segment substitution lines (CSSLs) or backcrossed inbred lines (BILs), have been developed for genetic analysis (Yano, 2001; Ebitani *et al.*, 2005; Yamamoto *et al.*, 2009). In addition, as a consequence of improvements in the quantitation of photosynthesis, it is now possible to reduce measurement times while maintaining accuracy in the field. Therefore, it is now possible to conduct research programmes that are aimed at improving leaf photosynthesis genetically. Quantitative trait loci (QTLs) for leaf photosynthetic rate have been identified in sunflower (Herve *et al.*, 2001) and in rice (Teng *et al.*, 2004). However, information about QTLs for leaf photosynthesis is very limited and, therefore, no near-isogenic lines (NILs) to analyse genetic effects in detail are available. The limited information might be a consequence of the minimal variations in rates of photosynthesis among the parental varieties used for genetic analysis and inadequate understanding of the factors that contribute to differences in photosynthetic rate. If it was possible to evaluate differences in rates of photosynthesis and identify the traits that contribute to an elevated rate of photosynthesis among parental varieties, the newly developed methods and plant materials for genetic analysis could be exploited and available varieties of rice could be improved.

A high-yielding *indica* variety, Habataki, has 1.3- to 1.4-fold higher rates of maximum photosynthesis than a commercial *japonica* variety, Sasanishiki, from booting to the early ripening stage (Asanuma *et al.*, 2008). In a previous study, the approximate chromosomal regions that determine leaf photosynthetic rate were localized on chromosomes 4, 5, 8, and 11 using CSSLs derived from Sasanishiki and Habataki (Nito *et al.*, 2007*a*, *b*). It was also found that the same regions on chromosomes 4 and 8 showed a larger hydraulic conductance (Asanuma *et al.*, 2007). The eventual goal of the present research is the improvement of the rate of leaf photosynthesis in the high-quality Japanese rice variety Koshihikari, which is currently the most widely farmed rice variety in Japan, by introgression of the chromosome segments of Habataki. However, the differences in the rate of photosynthesis and its related traits between Koshihikari and Habataki have not been determined. In addition to this, it is unclear whether the chromosomal regions of Habataki detected by Nito *et al.* (2007*a*, *b*) will increase the rate of leaf photosynthesis of Koshihikari or not. Populations were developed for genetic analysis with a focus on the regions of chromosome 4 and 8, where both the rates of leaf photosynthesis and hydraulic conductance were large among CSSLs from Sasanishiki and Habataki (Nito *et al.*, 2007*a*, *b*; Asanuma *et al.*, 2007). It was confirmed that these regions could increase the rate of photosynthesis in Koshihikari, the precise locations of the regions were determined, and then these regions were characterized based on analysis of the traits responsible for the differences in photosynthetic rate between Koshihikari and Habataki.

Materials and methods

Cultivation of rice plants

Rice plants of the *japonica* cultivar Koshihikari, the *indica* cultivar Habataki, and the mapping populations mentioned below were grown in the paddy field of the University Farm (35°41'N, 139°29'E). Seedlings at the fourth-leaf stage were transplanted to the paddy field in alluvial soil (clay loam) at a rate of 22.2 hills m^{-2} (spacing, 30 cm×15 cm) with one plant per hill. As a basal dressing, manure was applied at a rate of ~15 t ha^{-1} and chemical fertilizer was applied at a rate of 30, 60, and 60 kg ha^{-1} for N, P_2O_5, and K_2O, respectively. One-third of the total nitrogen was applied as nitrogen sulphate; one-third as LP-50 elution-controlled urea (Chisso Asahi Fertilizer, Tokyo); and one third as LPS-100 elution-controlled urea (Chisso Asahi Fertilizer). No topdressing was applied. Nitrogen was also applied at several different rates to Koshihikari and Habataki to change the levels of leaf nitrogen at the booting stage. The experiments were designed with five randomly arranged replicates.

Rice plants were also grown in 3.0 l pots that were filled with a mixture of paddy soil and Kanto diluvial soil (1:1, v/v) in a growth chamber (Koitotron, Koito Manufacturing Co. Ltd, Tokyo) or outdoors. Environmental conditions in the growth chamber were maintained at a day/night temperature of 28 °C/23 °C, a relative humidity of 60%/80%, a 12 h photoperiod, and a photosynthetic photon flux density (PPFD) at the top of the canopy of ~1000 μmol m^{-2} s^{-1}. Basal fertilizer was applied to the plants grown in a growth chamber at a rate of 0.5, 0.5, and 0.5 g per pot for N, P_2O_5, and K_2O, respectively, and 0.1 g per pot for N was applied to Koshihikari 10 d before measurements, depending on the reading of a chlorophyll meter (SPAD-502, Konica-Minolta, Tokyo), to bring the leaf nitrogen content of Koshihikari to the same as that of Habataki. Basal fertilizer was applied to the plants grown outdoors at a rate of 0.1, 0.5, and 0.5 g per pot for N, P_2O_5, and K_2O, respectively, and additional fertilizer was applied at a rate of 0.3 g of nitrogen per pot at the booting stage.

Rates of photosynthesis and stomatal conductance of individual leaves

The rate of photosynthesis (P_n) and the stomatal conductance (g_s) of the flag leaf were measured with portable gas-exchange systems (LI-6400 and LI-6200; LI-COR, Lincoln, NE, USA) at the full heading stage. The ambient CO_2 concentration in the leaf chamber of LI-6400 was kept at 370 μl l^{-1}, the PPFD was 2000 μmol m^{-2} s^{-1}, the leaf to air vapour pressure difference was 1.5–1.8 kPa on average, and the leaf temperature was 28 °C (for plants in the growth chamber) and 30 °C (for plants in the field) during measurements. P_n was also measured at different values of C_i by changing the ambient CO_2 concentration. The C_i was calculated as described by von Caemmerer and Farquhar (1981). Measurements with the LI-6200 system were started at an air CO_2 concentration of ~370 μl l^{-1} in the assimilation chamber. Measurements, for 8 s, were repeated three times and mean values were taken as the measured values. Leaves were exposed to natural light at a PPFD of >1200 μmol m^{-2} s^{-1} or to light from an artificial lamp (LA-180Me, Hayashi Watch Works, Tokyo) when the PPFD of sunlight was <1200 μmol m^{-2} s^{-1}. In the paddy field, plants were examined from 08:30 to 11:00 when P_n was close to the daily maximum (Hirasawa and Ishihara, 1992; Ishihara, 1995). The P_n and g_s of individual leaves were measured for 2 d or 3 d in succession and an average value was calculated.

Quantitation of the Rubisco content and the nitrogen content of individual leaves

Flag leaves were collected immediately after completion of measurement of P_n and g_s, and they were stored at –80 °C prior to analysis. The area and fresh weight of each leaf were determined and each leaf was separated into two equal parts for separate quantification of Rubisco and nitrogen. The halves of leaves were homogenized separately with a mortar and pestle in a solution that contained 50 mM TRIS-HCl (pH 7.5), 1 mM EDTA, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 5% (w/w) insoluble polyvinylpyrrolidone (Polyclar VT, Wako Chemicals, Tokyo). Each homogenate was centrifuged at 10 000 *g* for 10 min at 4 °C. The supernatant was used for quantitation of Rubisco by the single radial immunodiffusion method (Sugiyama and Hirayama, 1983) with rabbit polyclonal antibodies raised against purified Rubisco from rice. Nitrogen was quantified with a carbon and nitrogen analyser (MT700 Mark II, Yanako, Kyoto). Both Rubisco and nitrogen content of leaves were expressed on a per leaf area basis. In experiments in which Rubisco was not quantified, an 8 cm wide segment was cut from the central part of the leaf for quantitation of nitrogen.

Quantitation of the accumulation and distribution of nitrogen

Two plants with an average number of panicles per replicate were harvested in the paddy field at the full heading stage. Each plant was separated into leaves, stems plus leaf sheaths, and panicles, and dried in an oven at 80 °C for >72 h. After weighing, samples were powdered with a mill (WB-1, Osaka Chemical Ltd, Osaka) and the concentration of nitrogen in each sample was determined with the CN analyser (MT700 MarkII). The nitrogen content of each organ was determined by the product of the dry weight and the concentration of nitrogen.

Determination of the hydraulic conductance and hydraulic conductivity of plants

For plants grown in the paddy field, the hydraulic conductance from the soil through the roots to the flag leaf (C_p) was calculated from the following equation (Hirasawa and Ishihara, 1991):

$$C_p = T/(\Psi_s - \Psi_l) \quad (1)$$

where Ψ_s is the water potential of the soil immediately outside the root, Ψ_l is the water potential of a flag leaf, and T is the transpiration rate per leaf area at steady state. Since rice plants were grown under submerged conditions and the water potential of soil solution was high enough when compared with Ψ_l and kept constant, Ψ_s could be regarded here as 0. The transpiration rate of a single intact leaf was measured in an air-sealed acrylic assimilation chamber (Hirasawa and Ishihara, 1991) under natural sunlight. Air, with the dewpoint controlled to 10 (±0.1) °C, was pumped into the chamber at a rate of 6.67×10^{-5} m^3 s^{-1}. The humidity of the air that was pumped into and out of the chamber was measured with a dewpoint hygrometer (model 660, EG&G Inc., Waltham, MA, USA). When the transpiration rate reached a constant value, the water potential of the leaf was measured with a pressure chamber (model 3005, Soil Moisture Equipment Inc., Santa Barbara, CA, USA), as described by Hirasawa and Ishihara (1991). The transpiration rate was calculated per leaf area.

For plants grown in 3.0 l pots, C_p from roots to leaves was calculated as follows:

$$C_p = U_W/\Psi_l \quad (2)$$

where U_w is the water uptake rate of the whole plant and Ψ_l is the water potential of the uppermost three leaves. Measurements were made in an environment-controlled chamber (air temperature 28 °C, air vapour pressure deficit ~1.5 kPa, and PPFD at the top leaves ~1000 mol m^{-2} s^{-1}). The water uptake rate was determined from the rate of weight loss of the pot after a steady state had been reached. To prevent evaporation from the surface of the pot, the top of the pot was covered with polystyrene foam and oily clay was used to seal the gap between the foam and the stem. After measurement of the water uptake rate, the leaf water potential of the uppermost three leaves was measured with the pressure

chamber. Following that, roots were washed gently with water and the root surface area (A) was measured with an image analyser (Win-Rhizo REG V 2004 b, Regent Inc., Quebec, Canada). Hydraulic conductance per root surface area, taken here as the hydraulic conductivity of a plant (L_p), was calculated as follows:

$$L_p = C_p/A \quad (3)$$

Treatments with a polyethylene bag and leaf excision

To increase the stomatal conductance of the leaves of Koshihikari, polyethylene bag and leaf excision treatments were conducted. For polyethylene bag treatment, individual leaves were covered with a transparent polyethylene bag and illuminated with artificial light (LA-180Me) for 20 min. The PPFD at the surface of the bag was ~1000 μmol m^{-2} s^{-1}. Immediately after removal of the bag and blotting of water drops with gauze, P_n of the leaf was measured with an LI-6400 system at a CO_2 concentration of 370 μl l^{-1}. For leaf excision, after the leaf gas exchange had reached a steady state, the leaf was excised at its base with a razor blade. P_n was recorded continuously after both treatments at 5 s intervals and the maximum value was determined as the value 'after treatment'.

Plant materials for genetic analysis

Backcross progeny populations and lines derived from a cross between Koshihikari and Habataki were developed for genetic dissection of the regions for the rates of photosynthesis on chromosomes 4 and 8. BC_1F_1 that was crossed between F_1 and Koshihikari was developed. A total of 137 uniformly (average 10 cM) distributed simple sequence repeat (SSR) markers were used to determine the genotypes. In the following generations, the regions except the homozygous regions for the Koshihikari allele were determined by the SSR markers.

First, two BC_5F_1 plants were developed, 611-8 F_1 and 611-10 F_1, with a heterozygous genomic region on the long arm of chromosome 4 and on the short arm of chromosome 8, respectively. These regions included the QTLs for the photosynthetic rate defined in a previous report (Nito *et al.*, 2007*a*, *b*). 611-8 F_1 also carried heterozygous genomic regions on chromosomes 1, 2, and 5, as did 611-10 F_1 on chromosomes 2 and 5. One hundred plants from self-pollinated F_2 populations derived from each F_1 plant were transplanted to the paddy field.

Secondly, homozygous recombinant lines were developed to determine the precise locations of each QTL. Nine lines of BC_4F_3 or BC_5F_3 for identification of the region for chromosome 4 and nine lines of BC_5F_3 or BC_5F_4 for that on chromosome 8 were selected. Each line had only a single chromosomal segment from Habataki on the genetic background of Koshihikari.

QTL analysis

Total DNA from each plant of the 611-8 F_2 and 611-10 F_2 populations was extracted from leaves by the cetyltrimethyl ammonium bromide (CTAB) method (Murray and Thomson, 1980). The genotypes of chromosomes 4 and 8 were determined with seven and five SSR markers, respectively. Linkage maps were constructed using MAPMAKER/EXP 3.0 (Lander *et al.*, 1987). The positions on chromosomes and the effects of putative QTLs were determined by composite interval mapping with QTL Cartographer 2.0 (Basten *et al.*, 2002). The threshold for detection of a QTL was based on 1000 permutation tests at the 5% level of significance (Churchill and Doerge, 1994). The additive and dominant effects and phenotypic variance explained by each QTL were estimated at the peak of the log of the odds score (LOD).

Statistical analysis

The statistical significance of differences between two groups and between Koshihikari and other lines was determined by Student's t-test and by Dunnett's test, respectively, using KyPlot software (version 4.0, Kyence Tokyo).

Results

Characterization of the traits that influence the rate of leaf photosynthesis in Koshihikari and Habataki

At the full heading stage, the leaf photosynthetic rate (P_n) at an ambient CO_2 concentration of 370 μl l^{-1} in Habataki was very much higher than in Koshihikari in a submerged paddy field, when both cultivars were treated with the same fertilizer regime (Fig. 1A). Stomatal conductance (g_s) in Habataki was significantly higher than in Koshihikari (Fig. 1B). In addition, the Rubisco content of individual leaves was higher in Habataki than in Koshihikari, indicating that the photosynthetic activity of Habataki was higher than that of Koshihikari (Fig. 1C). Habataki also had a higher level of leaf nitrogen, which might be expected to contribute to the higher level of Rubisco (Fig. 1D). From these results, the higher stomatal conductance and the higher nitrogen content of individual leaves in Habataki were potential factors that contributed to the higher rate of photosynthesis. The total amount of accumulated nitrogen was significantly larger in Habataki than in Koshihikari, while the percentage of nitrogen distributed to leaves was somewhat smaller in Habataki

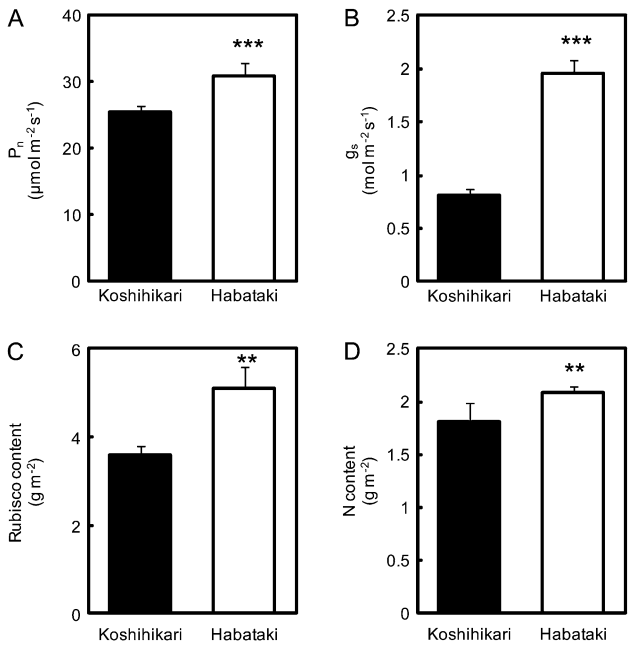


Fig. 1. Comparison of the rates of photosynthesis (P_n; A), stomatal conductance (g_s; B), Rubisco contents (C), and nitrogen (N) contents (D) of flag leaves of Koshihikari and Habataki after growth in the paddy field under submerged conditions to the full heading stage. P_n and g_s were measured with an LI-6400 system between 08:30 and 11:00. Vertical bars represent the SD (n=5). Asterisks ** and *** indicate significance at the 0.01 and 0.001 level, respectively, as compared with Koshihikari (t-test).

than in Koshihikari (Fig. 2). These findings indicated that the greater accumulation of nitrogen in Habataki than in Koshihikari was responsible for the higher level of leaf nitrogen.

In order to eliminate the effects of the difference in leaf nitrogen content on the difference in P_n, a small amount of nitrogen fertilizer was applied to Koshihikari and the leaf nitrogen content of Koshihikari was brought to that of Habataki (Table 1). Although there was no difference between Koshihikari and Habataki in terms of the relationship between the intercellular CO_2 concentration and P_n, P_n in Habataki at an ambient CO_2 concentration of 370 μl l^{-1} was still higher than that in Koshihikari due to the larger g_s of Habataki (Fig. 3). After leaves had been covered with transparent polyethylene bags for 20 min under an irradiance of ~1000 μmol photons m^{-2} s^{-1}, P_n in Koshihikari increased to the level in Habataki as a result of significant increases in g_s because of high humidity and low CO_2 concentration inside the bag, while the increases of P_n in Habataki were small (Table 1). The water potential of the flag leaf in Koshihikari decreased significantly compared with that in Habataki despite the fact that plants of both cultivars were growing under submerged soil conditions and the air vapour pressure deficit was as low as 1.5 kPa (Table 1). After excision of a leaf at its base and release of the hydrostatic pressure in the xylem, after leaf gas exchange had reached a steady state, P_n in Koshihikari increased to that in Habataki within a few minutes, a phenomenon known as the 'Ivanov effect' (Slavik, 1974), while there was little increase in Habataki (Table 1). The hydraulic conductance from roots to leaves (C_p) was far higher in Habataki than in Koshihikari (Table 1). Maintenance of the high water potential in Habataki, when stomata were fully open and the transpiration rate was high, was due to the higher hydraulic conductance of Habataki plants.

QTLs responsible for the rate of photosynthesis in leaves

To define the map positions of the QTLs that were identified on chromosomes 4 and 8 by use of CSSLs derived from Sasanishiki and Habataki in a previous study (Nito *et al.*, 2007*a*, *b*), QTL analysis was conducted for P_n using two BC_5F_2 populations derived from a cross between Koshihikari and Habataki at the full heading stage (Table 2). The primary generations, 611-8 F_1 and 611-10 F_1, had

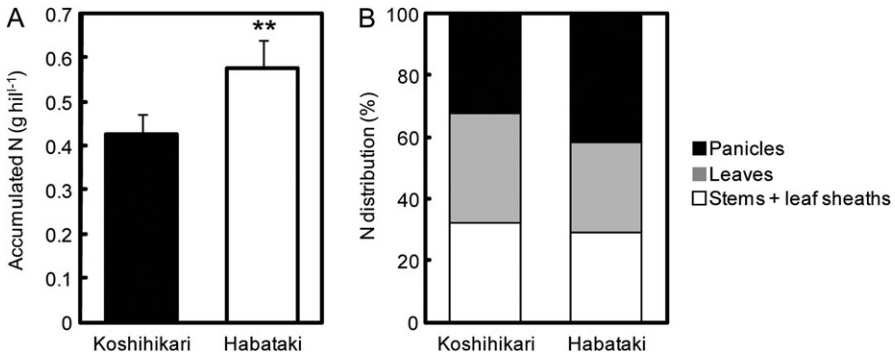


Fig. 2. Accumulated nitrogen in aboveground parts (A) and the nitrogen (N) distribution rates to panicles, leaves, and stems plus leaf sheaths (B) in Koshihikari and Habataki after growth in the paddy field to the full heading stage. Asterisks ** indicate significance at the 0.01 level as compared with Koshihikari (*t*-test).

Table 1. Changes in the rate of photosynthesis (P_n; μmol m^{-2} s^{-1}) after treatment with a polyethylene bag (A) and after excision (B), the hydraulic conductance from roots to leaves (C_p), and the leaf nitrogen (N) content in Koshihikari and Habataki that had been grown in 3.0 l pots under submerged conditions to the full heading stage

Leaf nitrogen contents in the two cultivars were equalized by application of ammonium sulphate to Koshihikari 10 d before measurements. Leaves in (A) were covered with a transparent polyethylene bag and illuminated for 20 min at a PPFD of 1000 μmol m^{-2} s^{-1} at the surface of the bag. Leaves in (B) were excised after P_n had reached a steady state. P_n increased for a few minutes, known as the 'Ivanov effect', and then decreased. The maximum P_n after both treatments was represented as P_n after treatment. C_p was expressed on a per leaf area basis. The water potential of an entire flag leaf blade, as measured with a pressure chamber, was –0.42±0.04 MPa and –0.32±0.03 MPa for Koshihikari and Habataki, respectively, at an air vapour pressure deficit of ~1.5 kPa, an air temperature of 28 °C, and a PPFD of 1000 μmol m^{-2} s^{-1}. Data are means ±SD (n=3–5). *, ** and *** indicate significance at the 0.05, 0.01, and 0.001 level, respectively, as compared with Koshihikari (t-test). NS, no significant difference.

	A		B		C_p (10^{-6} m MPa^{-1} s^{-1})	N content (g m^{-2})
	Before treatment	After treatment	Before treatment	After treatment		
Koshihikari	16.5±0.7	18.0±0.7	17.1±1.3	20.1±1.5	0.17±0.01	1.31±0.03
Habataki	19.0±1.2	18.9±1.1	20.7±0.7	21.1±1.1	0.29±0.02	1.24±0.11
	**	NS	*	NS	***	NS

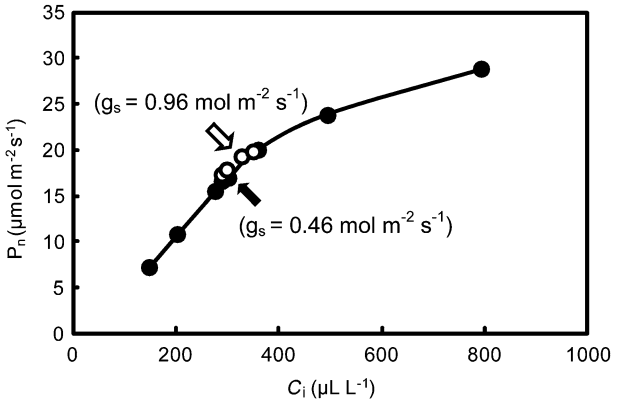


Fig. 3. Rates of photosynthesis (P_n) plotted against the intercellular CO_2 concentration (C_i) of the flag leaf with similar nitrogen content in Koshihikari (filled circles) and Habataki (open circles) after growth in 3.0 l pots under submerged conditions to the full heading stage. The filled (Koshihikari) and open (Habataki) arrows indicate rates at an ambient CO_2 concentration of 370 μl l^{-1}. The measurements were made with an LI-6400 system.

Table 2. Putative QTLs controlling the leaf photosynthetic rate detected in the BC_5F_2 populations grown in the paddy field at the full heading stage

The QTLs on chromosome 4 and 8 were detected in the 611-8 F_2 and 611-10 F_2 populations, respectively. The photosynthetic rate was measured with an LI-6200 system at an ambient CO_2 concentration of 370 μl l^{-1} between 08:30 and 11:00.

Trait	Chromosome	Flanking marker	LOD[a]	A[b]	D[c]	R^{2d}
Photosynthetic rate	4	RM3836	2.4	1.3	0.0	9.1
	8	RM6999	5.1	1.7	0.0	6.2

[a] A putative QTL with significant LOD scores in 1000 permutation tests at the 5% level.
[b] Additive effect of the allele from Habataki compared with that from Koshihikari.
[c] Dominant effect of the allele from Habataki compared with that from Koshihikari.
[d] Percentage phenotypic variance explained by each QTL.

heterozygous regions on the long arm of chromosome 4 and on the short arm of chromosome 8, respectively. From the analysis in 611-8 F_2, one QTL for increased P_n was identified at marker RM3836 on chromosome 4 (Table 2 and Fig. 4A). The phenotypic variance explained by the QTL was 9.1%. The additive effect of the Habataki allele was 1.3 μmol m^{-2} s^{-1}, indicating that the Habataki allele increased P_n. From the analysis of 611-10 F_2, a QTL for increased P_n was identified at marker RM6999 on chromosome 8 (Table 2 and Fig. 4C). The phenotypic variance explained by the QTL was 6.2%. The additive effect of the Habataki allele was 1.7 μmol m^{-2} s^{-1}, indicating that the Habataki allele increased P_n. From these results, it was confirmed that the chromosome segment of Habataki, which previously had an effect on the increase in the rate of photosynthesis of Sasanishiki, could also increase the rate of photosynthesis of Koshihikari.

Given the results of the QTL analysis, two sets of homozygous recombinant lines were developed, in which each line carried a single chromosomal segment from Habataki around the regions of the putative QTLs. All other regions of the genome were homozygous for the Koshihikari allele. In the case of the regions from chromosome 4, values of P_n at the full heading stage in T4-6, T4-7, and T4-8 were 14–16% higher than in Koshihikari, and the differences are significant (Fig. 4B). Substitution mapping, using nine lines, allowed the region to be narrowed down to an interval of ~2.1 Mb between RM17459 and RM17552. Similarly, in the case of the regions from chromosome 8, values of P_n in T8-3, T8-4, T8-5, T8-6, T8-7, and T8-8 were 21–28% higher than in Koshihikari and it was possible to narrow down the region to the interval of ~1.2 Mb between RM6999 and RM22529 (Fig. 4D). The reason why the P_n of these six lines increased comparably with that of Habataki is unclear.

Functions of the candidate genomic regions

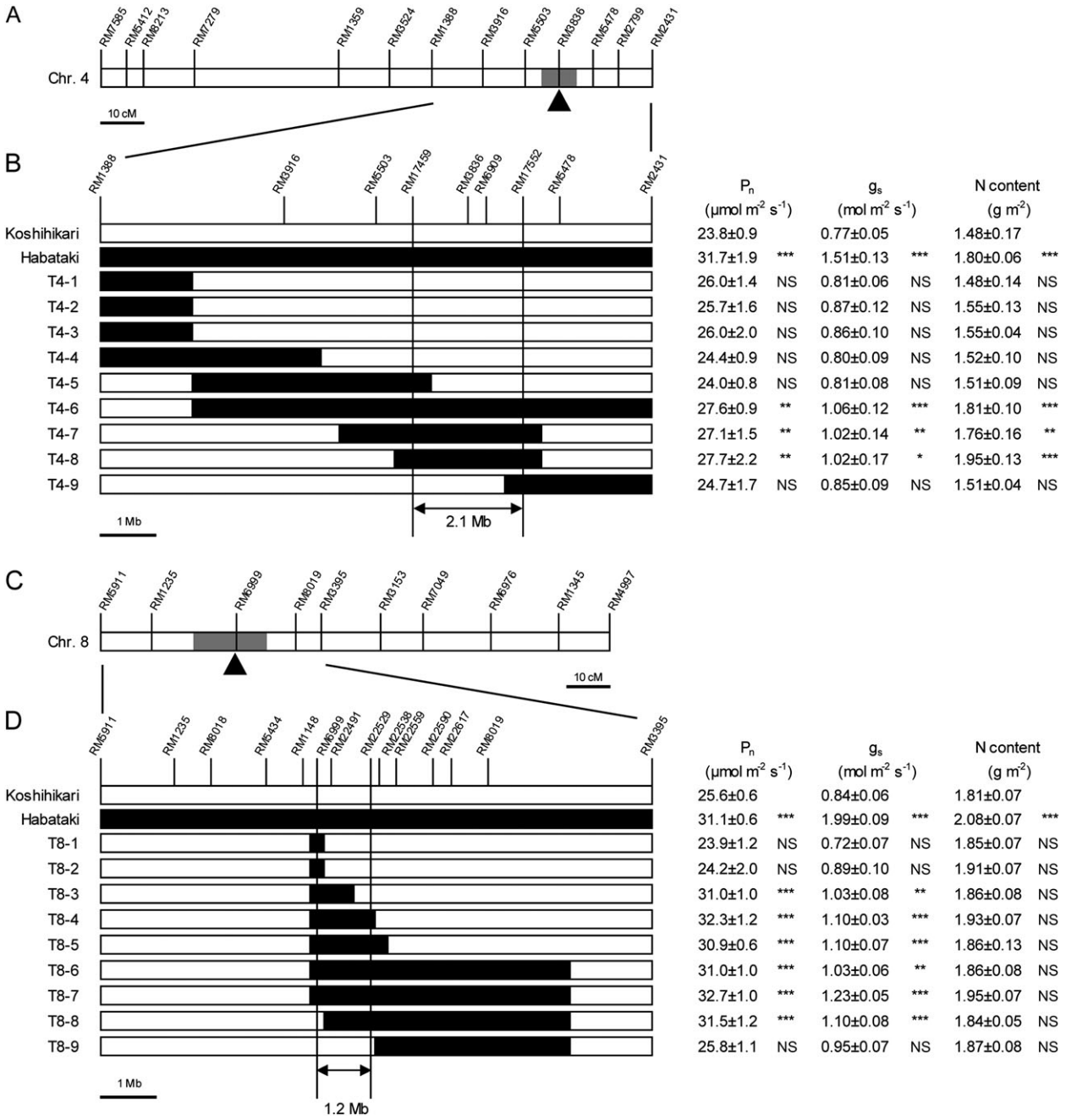
In the case of QTLs from chromosome 4, the lines with high values of P_n, namely T4-6, T4-7, and T4-8, had higher values of g_s and higher leaf nitrogen contents than Koshihikari (Fig. 4B). In contrast, with respect to chromosome 8, the leaf nitrogen contents in T8-3–T8-8, in which P_n were higher than in Koshihikari, were similar to those in Koshihikari, while values of g_s in these lines were higher than in Koshihikari (Fig. 4D).

T4-8 and T8-3 of these lines were considered as NILs and they wer used for more detailed analysis of the functions of these QTLs. It was found that both the P_n and g_s in T4-8 and T8-3 were higher than in Koshihikari when compared at the same leaf nitrogen content (Fig. 5). C_p in T4-8 and T8-3 was higher than that in Koshihikari (Fig. 6). The causes of the higher C_p in these lines were analysed by comparing root surface area and L_p (Table 3). Root surface area was higher in Habataki than in Koshihikari, while there is no difference in L_p between Koshihikari and Habataki. The larger root surface area might be responsible for the higher C_p of Habataki. The root surface area in T4-8 was ~30% larger than in Kohishikari and the L_p in T4-8 was similar to that in Koshihikari. In contrast, root surface area in T8-3 was as large as that in Koshihikari, but L_p in T8-3 was significantly higher than in Koshihikari.

Discussion

Traits contributing to the higher rate of photosynthesis in Habataki than in Koshihikari

In general, the rate of leaf photosynthesis in rice plants is affected to a significant extent by the carboxylation rate and the rate of diffusion of CO_2 from the atmosphere into the leaf at the ambient atmospheric concentration of CO_2 (Makino *et al.*, 1984; Kramer and Boyer, 1995). In the present study, a high-yielding *indica* variety, Habataki, had a higher rate of leaf photosynthesis than the *japonica* variety, Koshihikari (Fig. 1). The higher rate was caused by



	P_n (μmol m^{-2} s^{-1})		g_s (mol m^{-2} s^{-1})		N content (g m^{-2})	
Koshihikari	23.8±0.9		0.77±0.05		1.48±0.17	
Habataki	31.7±1.9	***	1.51±0.13	***	1.80±0.06	***
T4-1	26.0±1.4	NS	0.81±0.06	NS	1.48±0.14	NS
T4-2	25.7±1.6	NS	0.87±0.12	NS	1.55±0.13	NS
T4-3	26.0±2.0	NS	0.86±0.10	NS	1.55±0.04	NS
T4-4	24.4±0.9	NS	0.80±0.09	NS	1.52±0.10	NS
T4-5	24.0±0.8	NS	0.81±0.08	NS	1.51±0.09	NS
T4-6	27.6±0.9	**	1.06±0.12	***	1.81±0.10	***
T4-7	27.1±1.5	**	1.02±0.14	**	1.76±0.16	**
T4-8	27.7±2.2	**	1.02±0.17	*	1.95±0.13	***
T4-9	24.7±1.7	NS	0.85±0.09	NS	1.51±0.04	NS

	P_n (μmol m^{-2} s^{-1})		g_s (mol m^{-2} s^{-1})		N content (g m^{-2})	
Koshihikari	25.6±0.6		0.84±0.06		1.81±0.07	
Habataki	31.1±0.6	***	1.99±0.09	***	2.08±0.07	***
T8-1	23.9±1.2	NS	0.72±0.07	NS	1.85±0.07	NS
T8-2	24.2±2.0	NS	0.89±0.10	NS	1.91±0.07	NS
T8-3	31.0±1.0	***	1.03±0.08	**	1.86±0.08	NS
T8-4	32.3±1.2	***	1.10±0.03	***	1.93±0.07	NS
T8-5	30.9±0.6	***	1.10±0.07	***	1.86±0.13	NS
T8-6	31.0±1.0	***	1.03±0.06	**	1.86±0.08	NS
T8-7	32.7±1.0	***	1.23±0.05	***	1.95±0.07	NS
T8-8	31.5±1.2	***	1.10±0.08	***	1.84±0.05	NS
T8-9	25.8±1.1	NS	0.95±0.07	NS	1.87±0.08	NS

Fig. 4. QTL analysis and substitution mapping of genes for the leaf photosynthetic rate on chromosomes 4 and 8. (A and C) Chromosomal locations of QTLs that control the rate of photosynthesis. The open bars indicate the entire chromosomes. SSR markers used in the QTL analysis are indicated above the bars. The shaded bars indicate the confidence intervals of the QTLs detected by analysis of the BC_5F_2 populations. The arrowheads indicate the most likely position of the QTLs, as determined by composite interval mapping. (B and D) Substitution mapping of the QTLs that control leaf photosynthesis. The genotypes of Koshihikari, Habataki, and homozygous recombinant lines are shown schematically on the left. SSR markers used are indicated at the top of each panel. White bars, homozygous for Koshihikari alleles; black bars, homozygous for Habataki alleles. All other chromosome regions of the lines, which were not shown in the figure, were homozygous for Koshihikari alleles. On the right, the rate of photosynthesis (P_n), stomatal conductance (g_s), and the nitrogen (N) content of flag leaves of plants after growth in the paddy field to the full heading stage. Data are means ±SD (n=5). The measurements of P_n and g_s were made with an LI-6200 system (C) and an LI-6400 system (D) at an ambient CO_2 concentration of 370 μl l^{-1} between 08:30 and 11:00. The candidate genomic regions for the control of the rate of photosynthesis are indicated by double-headed arrows below the genotypes. Asterisks *, ** and *** indicate significance at the 0.05, 0.01, and 0.001 level, respectively, as compared with Koshihikari (Dunnett's test). NS, no significant difference.

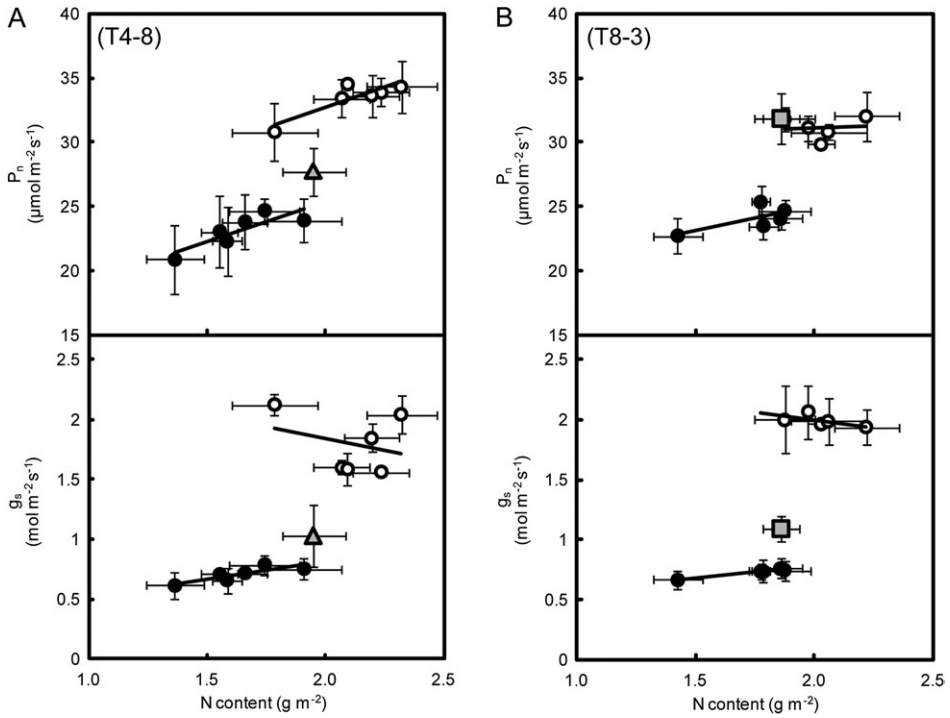


Fig. 5. Relationships between nitrogen content (N) and the rate of photosynthesis (P_n; upper panels) and stomatal conductance (g_s; lower panels) of flag leaves of homozygous recombinant lines and parental cultivars after growth in the paddy field to the full heading stage. Filled circles, open circles, shaded triangles (in A) and shaded squares (in B) represent Koshihikari, Habataki, T4-8, and T8-3, respectively. The measurements in (A) were made with an LI-6200 system and those in (B) were made with an LI-6400 system at an ambient CO_2 concentration of 370 μl l^{-1} between 08:30 and 11:00. Vertical and horizontal bars represent the SD (n=5).

a higher leaf nitrogen content and, therefore, a higher Rubisco content at a given rate of nitrogen application, as well as the greater stomatal conductance of each leaf at a given level of leaf nitrogen (Figs 3, 5).

Leaf nitrogen content

The leaf nitrogen content is determined by the net accumulation of aboveground nitrogen and the rate of distribution of nitrogen to leaves (Mae and Ohira, 1981). Varietal differences in both of these parameters have been reported (Ookawa *et al.*, 2003; Taylaran *et al.*, 2009). The rate of distribution of nitrogen to leaves was somewhat lower in Habataki than in Koshihikari, implying that the greater accumulation of nitrogen was responsible for the higher amount of nitrogen in Habataki leaves (Fig. 2). Root surface area and root length in Habataki were much higher than those in Koshihikari (Table 3), and it has been demonstrated that total root length and the accumulation of nitrogen are closely correlated in several old and new rice varieties (Taylaran *et al.*, 2009). In addition, differences in the activities of ammonium transporters in roots (Sonoda *et al.*, 2003; Suenaga *et al.*, 2003) and/or in the activities of enzymes such as glutamine synthetase and glutamate synthetase (Obara *et al.*, 2001) might contribute to differences in rates of nitrogen uptake per root surface area. The accumulation of nitrogen in plants is affected not only by the capacity of roots for nitrogen assimilation but also by the transportation of nitrogen to shoots via the transpirational stream (Cernusak *et al.*, 2009). The transpirational uptake of water is likely to be greater in plants with larger root systems (Hirasawa *et al.*, 1992*b*), and such uptake provides a plausible explanation for the greater accumulation of nitrogen by Habataki. However, the nitrogen assimilation capacity of the roots in Habataki remains to be investigated.

Stomatal conductance

The vapour pressure deficit with respect to the atmosphere has a marked effect on the stomatal conductance of rice via the rate of transpiration. In general, the deficit reaches ~1.5–1.8 kPa between 09:00 and 10:00 on a clear day. It is well known that, even if soil moisture conditions are adequate, the rate of photosynthesis in leaves of rice plants decreases at midday and in the afternoon, as a result of water stress, after the rate has reached a maximum during the morning (Hirasawa and Ishihara, 1992; Ishihara, 1995), as is also true in maize (Hirasawa and Hsiao, 1999). However, the present results (Table 1) indicate that, even with such a relatively low vapour pressure deficit in the morning, the leaves of Koshihikari experience water stress, and P_n, measured in the morning, does not approach the maximum value.

The water balance within a plant is strongly affected by hydraulic conductance (Holbrook, 2006; Hopkins and Huner, 2008). With increases in transpiration, the leaf water potential decreases more in plants with low hydraulic

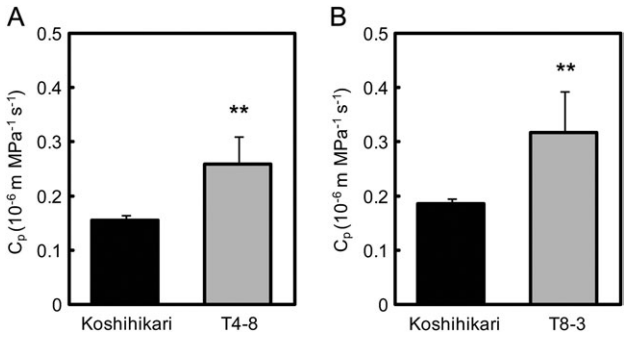


Fig. 6. Hydraulic conductance from roots to the flag leaf (C_p) in T4-8 (A) and T8-3 (B) compared with that in Koshihikari after growth in the paddy field to the ripening stage. C_p was expressed on a per leaf area basis. Vertical bars represent the SD (n=5). Asterisks ** indicate significance at the 0.01 level, as compared with Koshihikari (t-test).

Table 3. Hydraulic conductance of plants (C_p), root length, root surface area, and hydraulic conductivity of plants (L_p) grown in 3.0 l pots under submerged conditions to the ripening stage

Data are means ±SD (n=2– 5). C_p, root length and root surface area were expressed on a per stem basis. Asterisks *, ** and *** indicate significance at the 0.05, 0.01, and 0.001 levels, respectively, as compared with Koshihikari (Dunnett's test). NS, no significant difference.

	C_p (10^{-6} m^3 MPa^{-1} s^{-1})	Root length (m)	Root surface area (m^2)	L_p (10^{-8} m MPa^{-1} s^{-1})
Koshihikari	9.8±0.9	88.3±1.4	0.060±0.003	1.55±0.14
Habaraki	22.4±1.0***	211.8±8.8***	0.156±0.003***	1.40±0.11^{NS}
T4-8	11.5±1.0*	120.2±11.7*	0.077±0.006*	1.51±0.09^{NS}
T8-3	11.7±0.6*	81.0±7.9^{NS}	0.056±0.006^{NS}	2.17±0.10**

conductance than it does in plants with high hydraulic conductance (Slatyer, 1967). The critical water potential for stomatal closure is very much higher in rice than in other crop plants, such as wheat, soybean, and cotton (Hirasawa, 1999). These observations might explain why the hydraulic conductance affects stomatal conductance. Brodribb *et al.* (2007) found that, in an analysis of 43 plant species, the maximum rate of photosynthesis was correlated both with the distance between veins and the leaf surface and with the hydraulic conductance of the leaf. Woodruff *et al.* (2008) found, in the foliage of *Pseudotsuga menziesii* trees of different height classes, that the maximum rate of photosynthesis was correlated both with mesophyll thickness and with the hydraulic conductance of individual leaves. The present results confirmed the importance of hydraulic conductance in defining photosynthetic capacity.

The hydraulic conductance in entire rice plants is determined mainly by the conductance of the roots (Hirasawa *et al.*, 1992*a*). Since the axial conductance of roots is very large compared with the radial conductance, root hydraulic conductance can be considered to be derived from two components: root hydraulic conductivity (L_{pr}; conductance per root surface area) and root surface area (Steudle and Peterson, 1998). Root surface area in Habataki was much larger than that in Koshihikari, but there was no difference in L_p between Koshihikari and Habataki. It was concluded that the larger root surface area contributed to the higher C_p in Habataki (Table 3).

Chromosomal regions responsible for the rate of photosynthesis

Genetic regions that control the rate of photosynthesis were identified between RM17459 and RM17552 on chromosome 4 and between RM6999 and RM22529 on chromosome 8 (Fig. 5). Teng *et al.* (2004) identified QTLs for the rate of leaf photosynthesis on chromosomes 4 and 6 using a double haploid population derived from anther culture of ZYQ8/JX17. However, these regions are not identical to the regions that were located here. To our knowledge, no NILs with a high rate of photosynthesis were available and the genomic effects have not been analysed in detail. Two NILs carrying a QTL (i.e. QTL-NILs) were developed and the traits contributing to an increase in the rate of photosynthesis were analysed. According to the results, the QTL region on chromosome 4 increases leaf nitrogen content and g_s via increases in C_p through a larger root surface area, while the QTL region on chromosome 8 increases C_p through a larger L_p and, as a consequence, g_s (Figs 4–6; Table 3).

Genetic regions responsible for leaf nitrogen content

Ishimaru *et al.* (2001) identified QTLs responsible for leaf nitrogen content on chromosome 2 and for Rubisco content on chromosomes 8, 9, and 12 using backcross inbred lines derived from a cross between Nipponbare and Kasalath. Kanbe *et al.* (2009) detected several QTLs for Rubisco content on chromosome 10 using CSSLs and backcross progeny populations derived from a cross between Koshihikari and Kasalath. Talukder *et al.* (2005) identified QTLs for the nitrogen concentration in individual plants on chromosomes 1, 2, 3, 7, 10, 11, and 12 using recombinant inbred lines derived from a cross between Bala and Azucena. However, these QTLs did not overlap with those identified on chromosome 4 in the present study.

Genetic regions responsible for stomatal conductance and hydraulic conductance

Although there are several reports of QTLs that control g_s (Price *et al.*, 1997; Frei *et al.*, 2008; Khowaja and Price, 2008), these QTLs do not overlap the region identified in the present study. Many QTLs associated with root characteristics have been found close to the QTL on chromosome 4. QTLs for root thickness (Zhang *et al.*, 2001; Kamoshita, 2002; Price *et al.*, 2002), rooting ability of seedlings (Ikeda *et al.*, 2007), and the dry weight and volume of roots (Qu *et al.*, 2008) have been identified close to the region that was identified on chromosome 4. Thus, it is necessary to analyse this chromosomal region with the focus on the

relationships between various root-morphological traits and functions of roots for the uptake and transport of water.

Interestingly, L_p in T8-3 was found to exceed that of Habataki, while the root surface area did not increase (Table 3). Varietal differences in the root hydraulic conductivity (L_{pr}) in rice are not known (Table 4; Miyamoto *et al.*, 2001) and there are no reports of QTLs for L_{pr} in rice so far. The present finding suggests the possibility of improvement in C_p by increases of not only root surface area but also L_{pr} with marker-assisted selection. It will be necessary to examine in more detail the causes of the difference in L_{pr}.

The region of chromosome 4 was associated with increased nitrogen accumulation and water uptake, suggesting that separate traits controlled these two characteristics. However, given the correlation between root mass and the accumulation of nitrogen by the entire plant reported by Taylaran *et al.* (2009) and the relationship between root mass and hydraulic conductance of roots (Hirasawa *et al.*, 1992*b*), it can be postulated that the increase in both the ability to accumulate nitrogen and water uptake might be caused by development of the root system. Isolation and characterization of the responsible genes on both chromosomes 4 and 8 are necessary for detailed analysis of the mechanisms of increased photosynthesis.

To increase the rate of leaf photosynthesis of Koshihikari further, it is necessary to accumulate multiple QTLs associated with P_n. Experiments are now in progress to try to combine the QTLs of chromosomes 4 and 8 and to evaluate the combined effect on P_n. Two other QTLs were localized using CSSLs derived from a cross between Sasanishiki and Habataki in a previous study (Nito *et al.*, 2007*a*, *b*). The functions of the two QTLs in P_n and the effects of accumulation of all QTLs remain to be investigated.

Acknowledgements

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