Mapping of QTLs Controlling Carbon Isotope Discrimination in the Photosynthetic System using Recombinant Inbred Lines Derived from a Cross between Two Different Rice (Oryza sativa L.) Cultivars

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Abstract: Carbon isotope discrimination (\(\Delta\)) occurring in the process of photosynthesis, shows variation among rice (Oryza sativa L.) cultivars. Elucidation of specific traits associated with the extent of this discrimination under irrigated conditions may be useful to improve photosynthetic ability in rice plants. We measured leaf photosynthesis and \(\Delta\) in Milyang 23 and Akihikari, and conducted quantitative trait loci (QTL) analysis on \(\Delta\) at heading stage using a population of 126 recombinant inbred lines (RILs), derived from a cross between the two cultivars. While the two parental cultivars showed a similar \(\Delta\), the RILs showed a wide variation in \(\Delta\) including transgressive segregation. Seven QTLs were detected for \(\Delta\); four on chromosomes 2 (two regions), 7, and 11 were those for \(\Delta\) that is increased by the Milyang 23 allele, whereas the other three on chromosomes 1, 2, and 6 were those for \(\Delta\) that is increased by the Akihikari allele. These results suggest that \(^{13}\)C in Milyang 23 may be discriminated through a photosynthetic process different from that in Akihikari. Milyang 23 showed a higher stomatal conductance and a higher ratio of intercellular to ambient CO2 concentration (G/\(\text{Ca}\)), while Akihikari showed a higher carboxylation efficiency but a lower C/\(\text{Ca}\). According to the theory that a higher G/\(\text{Ca}\) leads to a higher \(\Delta\), the QTLs for \(\Delta\) that is increased by the Milyang 23 allele might be related to a higher stomatal conductance. However, the theory provided no persuasive factors to explain the QTLs for \(\Delta\) that is increased by the Akihikari allele. Plausible factors associated with these QTLs are discussed.

Key words: Carbon isotope discrimination (\(\Delta\)), Photosynthesis, Quantitative trait locus (QTL), Rice (Oryza sativa L.).

CO2 assimilation by leaf photosynthesis is vital for plant growth and development. Approximately 90% of the biomass of higher plants is derived from CO2 fixed by photosynthesis (Zelitch, 1982). In addition to the undoubted evidence that photosynthesates during the grain filling period directly contribute to the final grain yield in rice (Oryza sativa L.) plants (Yoshida, 1972), a higher leaf photosynthetic rate during the later reproductive period (14 to 0 d before full heading) is suggested to be prerequisite for the achievement of higher grain yield (Horie et al., 2003; Takai et al., 2005). Therefore, improvement of leaf photosynthesis may be one way to increase the yield potential of rice.

Leaf photosynthesis can be measured by the gas exchange system using a leaf chamber. Numerous studies revealed a high positive correlation between light-saturated photosynthesis and nitrogen content in leaves of rice plants, since nitrogen invested in photosynthetically associated enzymes in chloroplasts accounts for a substantial part of leaf nitrogen content (Sinclair and Horie, 1989; Peng et al., 1995; Makino, 2003). Also, a higher stomatal conductance has been associated with a higher leaf photosynthetic rate (Wong et al., 1979; Kuroda and Kumura, 1990). This relationship suggests the importance of the CO2 supply to the chloroplasts for the increased photosynthetic rate (Ishihara et al., 1979; Horie et al., 2003). However, since uniform weather conditions are needed to detect the genotypic variation of photosynthetic rate and stomatal conductance, it is too time-consuming and difficult to directly measure them in a large breeding population (Samejima, 1985; Amani et al., 1996; Condon et al., 2004).

In the process of photosynthesis, plants discriminate against the stable isotope of carbon (\(^{13}\)C) present in ambient CO2. Farquhar et al. (1982) proposed that the extent of this discrimination, called carbon isotope discrimination (\(\Delta\)), on a dry matter basis in C3 plants could reflect the mean ratio of intercellular to ambient CO2 concentration (G/\(\text{Ca}\)) over the period when the dry matter was formed

\[
\Delta = a + (b - a) \frac{G}{\text{Ca}}
\]
where $a$ is the discrimination that occurs during diffusion of CO$_2$ into the intercellular airspaces (4.4 ‰), and $b$ is the discrimination associated with carboxylation by Rubisco ($b_29$‰). Because the value of Ci/Ca is determined by the balance between stomatal conductance and carboxylation efficiency in the chloroplasts, $\Delta$ can also represent the result of the balance (Condon et al., 2002). The major advantage of using $\Delta$ is that stored samples can be used in the automated measurements (Condon et al., 1987), which is more suitable for the evaluation of a great number of plants than the gas exchange system. In spite of its expected utility, it is unclear whether variation in $\Delta$ is driven by variation in stomatal conductance or carboxylation efficiency (Condon et al., 2002; Earl, 2002).

The development of linkage mapping with DNA markers has promoted the detection of chromosome regions controlling quantitative traits, called quantitative trait loci (QTLs), toward the more effective breeding (Tanksley, 1993). Some studies have successfully revealed potential QTLs for $\Delta$ in rice (Ishimaru et al., 2001; Price et al., 2002), barley (Teulat et al., 2002), soybean (Specht et al., 2001), pasture legume (Thumma et al., 2001), and maritime pine (Brendel et al., 2002). However, the majority of QTLs detected in these studies resulted from arid experimental conditions, so that it is not clear whether those QTLs were derived from the intrinsic or stress-induced variation in $\Delta$. Furthermore, the traits of photosynthesis associated with those QTLs could not be determined in those studies. From the viewpoint of functional genomics as well as improvement of leaf photosynthesis, we need more information about the specific physiological traits involved in QTLs for $\Delta$.

### Materials and Methods

1. **Gas exchange measurements**

   1. **Cultivation of plant materials**

      Milyang 23 and Akihikari were sown in the seedling nursery on 4 May and transplanted into 1/5000a Wagner pots with one seedling per pot at Kyoto (35° 2’N, 135°47’E, 65 m altitude), Japan on 26 May in 2004. Milyang 23 is a high-yielding Korean Indica-type cultivar with heavy panicle. Akihikari is also regarded as a high-yielding Korean Indica-type cultivar with heavy panicle. Akihikari is also regarded as a high-yielding Japonica-type variety in Japan. As basal application, 0.3 g N, 1.4 g P, and 0.7 g K were applied per pot with one plant, and as topdressing, 0.2 g N per pot was applied two times; i.e., at tillering and reproductive stages. Until gas exchange measurements, plants were grown uniformly in outdoor conditions. Gas exchange was measured at the reproductive period (72 and 73 days after transplanting (DAT) in both cultivars) and at the heading stage (80 and 81 DAT in Akihikari and 96 and 97 DAT in Milyang 23).

### Table 1. Net leaf photosynthetic rate ($P_n$) and its associated traits in Milyang 23 and Akihikari at reproductive and heading stages in 2004.

|                      | $P_n$ (µmol m$^{-2}$ s$^{-1}$) | $g_{co2}$ (mol m$^{-2}$ s$^{-1}$) | Ci/Ca (mol m$^{-2}$ s$^{-1}$) | IS (µmol m$^{-2}$ s$^{-1}$) | $\Gamma^*$ (µmol m$^{-2}$ s$^{-1}$) | $\Delta$ (‰) | Leaf nitrogen content (× 100 g g$^{-1}$) |
|----------------------|-------------------------------|----------------------------------|-------------------------------|---------------------------|-----------------------------------|--------------|------------------------------------------|
| **reproductive stage**                            |                               |                                  |                               |                          |                                    |              |                                          |
| Milyang 23 (n=4)     | 29.8 ± 1.6$^b$                | 0.42 ± 0.04                      | 0.78 ± 0.01                   | 0.131 ± 0.006             | 42.0 ± 2.1                      | 21.61 ± 0.22 | 3.43 ± 0.17                              |
| Akihikari (n=6)      | 29.4 ± 1.0                    | 0.32 ± 0.02                      | 0.72 ± 0.01                   | 0.138 ± 0.002             | 41.5 ± 1.6                      | 21.44 ± 0.12 | 3.42 ± 0.12                              |
| $P$                  | n.s.                          | <0.05                            | <0.01                         | n.s.                      | n.s.                             |              |                                          |
| **heading stage**    |                               |                                  |                               |                          |                                    |              |                                          |
| Milyang 23 (n=6)     | 24.9 ± 0.9                    | 0.35 ± 0.02                      | 0.78 ± 0.01                   | 0.106 ± 0.004             | 39.1 ± 1.0                      | 21.22 ± 0.07 | 2.49 ± 0.09                              |
| Akihikari (n=5)      | 27.4 ± 0.6                    | 0.30 ± 0.02                      | 0.71 ± 0.01                   | 0.141 ± 0.003             | 38.3 ± 0.3                      | 21.46 ± 0.10 | 3.51 ± 0.05                              |
| $P$                  | <0.05                         | <0.10                            | <0.01                         | <0.01                     | n.s.                             |              |                                          |

$^a$ The number of plants measured.

$^b$ Values are shown as mean ± standard error.

$g_{co2}$: stomatal conductance, Ci/Ca: the ratio of intercellular to ambient CO$_2$ concentration, IS: initial slope of Ci-Pn curve, $\Gamma^*$: CO$_2$-compensation point, $\Delta$: carbon isotope discrimination.
Gas exchange system

Gas exchange was measured on topmost fully-expanded leaves using a laboratory-constructed gas exchange system. The system was composed of four acrylic chambers (30 × 5.5 × 6.5 cm) in which each individual leaf, attached to plants, was enclosed. The air in each chamber was circulated using four fans (Brushless, DC Fine Ace 20, SANYO, Japan) and the diffusion conductance of water vapor in leaf boundary layer was estimated to be 1.46 mol H$_2$O m$^{-2}$ s$^{-1}$. Air temperature inside the chamber was maintained at 30 ±1°C by circulating water around the chamber from an external temperature-controlled water bath. Leaf temperature was monitored with a copper-constantan thermocouple pressed against the abaxial surface of the leaf.

Twelve halogen lamps (JD500W-M, IWASAKI, Japan) above the chambers gave photosynthetically active photon flux density (PPFD) of 1400 µmol m$^{-2}$ s$^{-1}$ at the leaf surface measured with a quantum sensor (LI-190SH, LI-COR, USA). The temperatures and PPFD were continuously recorded on a hybrid recorder (DR232, YOKOGAWA, Japan).

The current system allowed us to regulate CO$_2$ concentration in the chambers. When gas exchange on the leaf was measured below the ambient CO$_2$ concentration, the compressed air was combined with 10% CO$_2$ in air using a mass-flow controller (Model 3660, KOFLOC, Japan). Relative humidity was adjusted at 70%, by passing CO$_2$-adjusted air through a humidifier, followed by a dehumidifier before entering the chamber.

The flow rate of air entering the four chambers was monitored with a mass-flow meter (Model RK1200, KOFLOC, Japan). Leaf assimilation and transpiration rates were calculated from the CO$_2$ depletion and H$_2$O increment in the air in the chamber, measured with a CO$_2$/H$_2$O analyzer (LI-7000, LI-COR, USA). Gas exchange parameters were calculated according to von Caemmerer and Farquhar (1981).

Experimental protocol

Plants were kept in the shade in the afternoon before gas exchange measurements. The following morning, rice plants were transferred to the laboratory, and a single-leaf of each of the four plants was enclosed in the chamber, separately. Each leaf in the four chambers was allowed to reach a steady state in photosynthesis under ambient CO$_2$ conditions. Subsequently, the CO$_2$ concentration was varied stepwise from 10 to 1500 ppm to obtain the CO$_2$ assimilation rate in a broad range of Ci values. If a significant drift in photosynthesis had occurred during the gas exchange measurements, we discarded the sample. Four to six plants of each cultivar and
at each developmental stage were measured. After gas exchange measurements finished, the leaf area was measured with an automatic area meter (AAM-7, Hayashi Co. Ltd., Tokyo, Japan). Then, the leaf sample was oven dried for measurements of carbon isotope composition (δp) and leaf nitrogen content. We examined δp by the continuous flow system for isotope analysis, a mass spectrometer (Delta-S, Thermoquest, USA) with an elementary analyzer (EA1108, FISON, Italy). We calculated ĉ from the following equation, assuming that carbon isotope composition of the air (δa) was −8 ‰.

\[ ĉ = \frac{δa - δp}{1 + \frac{δa}{1000}} \]  

Leaf nitrogen content was measured with the elementary analyzer.

2. QTL analysis for carbon isotope discrimination

(1) Plant materials and field experiments

A population of 126 RILs constructed by a single seed descent method from a cross between Milyang 23 and Akihikari (Fukuta et al., 2004) was used in this study. Field experiments were conducted at the International Rice Research Institute (IRRI) farm, Los Baños (14°11’N, 121°15’E, 21m altitude), Laguna, Philippines, during the wet season from June to October in 2002 and the dry season from January to May in 2003. In 2002, two parents and 126 RILs (F11 generation) sown on 29 June were transplanted with one seedling per hill with one replication on 24 July. Each plot consisted of 5 rows with 12 hills per row and hill spacing was 0.3 × 0.2 m. As basal fertilizer, we applied 30 kg N ha⁻¹, 30 kg P ha⁻¹, and 30 kg K ha⁻¹. Twenty kg N ha⁻¹ was applied as topdressing both at 2 and 4 weeks after transplanting. In 2003, 25-day-old seedlings of the parents and each RIL (F12) were transplanted with one seedling per hill with one replication on 11 February. Seven rows with 12 hills per row formed a plot and hill space was 0.2 × 0.2 m. The amount of fertilizer applied as basal dressing was 40 kg N ha⁻¹, 40 kg P ha⁻¹, and 40 kg K ha⁻¹. Forty kg N ha⁻¹ was topdressed at 2 wks after transplanting. In both years, the practical field management was performed for pests, insects, and water on the basis of IRRI experimental farm practices.

Heading stage was defined as the stage when 50% of hills had at least one stem which had started heading, and days-to-heading as the number of days from sowing to heading was counted. The flag leaf and the 2nd leaf on the main stem or primary tiller were collected from five and ten plants per plot at heading stage in 2002 and 2003, respectively. Leaf samples were dried in the oven at 70°C to a constant weight. Leaf samples of each plot were weighed, bulked and ground to a fine powder for the analysis of δp and leaf nitrogen content. δp was determined by the method mentioned above, and leaf nitrogen content was determined by the Kjeldahl method (Bremner and Mulvaney, 1982).

(2) QTL analysis

Using 192 restriction fragment length polymorphism (RFLP) markers and 81 simple sequence repeat (SSR) markers, Fukuta et al. (2004) determined the marker loci of F10 genotypes and constructed a linkage map (Fig. 3). The chromosomal positions and effects of putative QTLs were determined by composite interval mapping (CIM) using QTL Cartographer 2.0 (Basten et al., 2002) since CIM is well known to have the advantages over simple interval mapping in more precisely detecting the position and effects of the QTLs (Zeng, 1994). QTL cartographer’s Zmap QTL, Model 6
with a window size of 10 cM was used for CIM. The number of markers for the background control was set to 5. A likelihood ratio of 11.5, corresponding to a logarithm of odds (LOD) score of 2.5, was used as a threshold value to detect a putative QTL, since Yano and Sasaki (1997) pointed out that a higher threshold may underestimate putative QTLs and bias towards genes with larger phenotypic effects. To ensure the false positive (type I error) rate for QTL detection, we conducted 1,000 permutation tests for each trait at the 5% level of significance (Churchill and Doerge, 1994; Doerge and Churchill, 1996). The peak of LOD score with one-LOD support interval was accepted as the location of putative QTL (van Ooijen, 1992), and the additive effect and phenotypic variance explained by each QTL were estimated at the peak of LOD score.

### Results

1. **Leaf gas exchange in Milyang 23 and Akihikari**

   Table 1 shows the net leaf photosynthetic rate (Pn) and its associated traits in Milyang 23 and Akihikari at the reproductive and heading stages in 2004. Measured Pn was 14% higher in Akihikari than in Milyang 23 at the heading stage, while there was no significant difference at the reproductive stage. Leaf nitrogen content did not differ between the two cultivars at the reproductive stage, but it was significantly higher (P < 0.01) in Akihikari than in Milyang 23 at the heading stage. The initial slope of Ci-Pn curve (IS), obtained by linear regression at less than 300 μmol mol⁻¹ of CO₂ (Fig. 1), was higher (P < 0.01) in Akihikari than in Milyang 23 at the heading stage. A significant difference was observed in stomatal conductance (gₑ) at both stages. Milyang 23 showed 31 and 17% higher gₑ than Akihikari at reproductive and heading stages, respectively. Milyang 23 having higher gₑ also maintained a higher Ci/Ca than Akihikari at both stages. Although a significant difference in Ci/Ca was observed between the two cultivars, the difference in Δ was only 0.2 ‰ at both stages.

2. **Phenotypic variation in RILs**

   Three traits, days-to-heading, Δ, and leaf nitrogen content in the RILs showed continuous variation in both 2002 and 2003 (Fig. 2). Akihikari reached the heading stage earlier than Milyang 23; days-to-heading in Akihikari were 66 and 62 in 2002 and 2003, respectively, whereas those in Milyang 23 were 83 and 84, respectively. The Δ in Milyang 23 and Akihikari at heading stage was 21.30 and 21.58 ‰, respectively, in 2002, and 20.73 and 21.13 ‰, respectively, in 2003. The small difference in Δ between the two cultivars in the two years was similar to that in 2004. On the other hand, the Δ in RILs demonstrated transgressive segregation of about 3 ‰, ranging from 19.34 to 22.17 ‰ in 2002 and from 18.62 to 21.82 ‰ in 2003. An approximately 1% difference existed in leaf nitrogen content at the heading stage between Milyang 23 (2.96 and 2.68 % in 2002 and 2003, respectively) and Akihikari (4.08 and 3.82 % in 2002 and 2003, respectively) across the two years. This difference between the two cultivars at heading was also comparable to that in 2004.

   The days-to-heading was negatively correlated with Δ and leaf nitrogen content in RILs, but there was no significant correlation between Δ and leaf nitrogen content in either year (Table 2). A strong correlation (r = 0.78; P < 0.01) was observed in Δ between 2002 and 2003, indicating that Δ was a stable inherited trait under the irrigated field conditions. Although a significant correlation existed in leaf nitrogen content between the two years, the correlation coefficient was not high (r = 0.41; P < 0.01) compared to that of Δ.

3. **QTLs identified for the three traits**

   A total of ten and twelve QTLs significantly affecting days-to-heading, Δ, and leaf nitrogen content were detected in 2002 and 2003, respectively, using a LOD threshold of 2.5 (Table 3 and Fig. 3). The phenotypic
For days-to-heading, four QTLs on chromosomes 3 (two regions), 4, and 7 and three on chromosomes 2, 4, and 10 were identified in 2002 and 2003, respectively. The $R^2$ of these QTLs ranged from 6.9 to 16.9%. While the Milyang 23 allele of all QTLs detected in 2002 and two QTLs on chromosomes 2 and 4 in 2003 contributed to delay of heading, the only QTL on chromosome 10 in 2003 contributed to hasten it. There were no QTLs for days-to-heading detected repeatedly in the two years. Flowering time of rice plants is substantially controlled by photoperiod (Lin et al., 2000). Since day length between sowing and heading changed from 12.8 to 11.9 h in 2002 and from 11.3 to 12.4 h in 2003, different QTLs for days-to-heading may have been expressed under the different photoperiods in the two years.

Five and seven QTLs, explaining $R^2$ from 7.1 to 14.3%, were detected for $\Delta$ in 2002 and 2003, respectively. Among them, five QTLs at the short arm on chromosome 2 (two regions) and at the long arms on chromosomes 6, 7, and 11 were identified across the two years. Although two QTLs at the long arms on chromosomes 1 and 2 were detected only in 2003, the LOD peaks of 2.26 and 1.87, which were less than the threshold value of 2.5, existed at the same regions in 2002 (data not shown). It is of interest that the Milyang 23 alleles of four QTLs at the short arm on chromosomes 2 (two regions) and at the long arms on chromosomes 7 and 11 were associated with increased $\Delta$, while other three QTLs at the long arms on chromosomes 1, 2, and 6 positively affected $\Delta$ that is increased by the Akihikari alleles. Detected QTLs for $\Delta$ were not linked to those for days-to-heading, indicating that both traits may be controlled by different genetic regulations.

There was one QTL for leaf nitrogen content on chromosome 2 in 2002 and two on chromosome 1 in 2003 with $R^2$ ranging from 7.6 to 14.5%. The Akihikari allele of the QTLs in the vicinity of XNpb147 on chromosome 1 and RM324 on chromosome 2 contributed to increased leaf nitrogen content, but decreased it for the QTL close to RM104 on chromosome 2. The QTL detected near XNpb147 overlapped that for $\Delta$.

**Discussion**

A total of seven QTLs were detected for $\Delta$ on the whole chromosome regions in 2002 and 2003 (Table 3 and Fig. 3). Among these QTLs, five were repeatedly detected across the two years. This result indicates that $\Delta$ is a stable inherited trait under the irrigated field conditions in rice. Several QTLs have been reported

### Table 3. Putative QTLs controlling days to heading, carbon isotope discrimination ($\Delta$), and leaf nitrogen content in 2002 wet and 2003 dry seasons.

| Trait                        | 2002 wet season | 2003 dry season |
|------------------------------|-----------------|-----------------|
|                              | Chr.  | Franking markers | LOD  | $R^2$ | $A^c$ | Chr.  | Franking markers | LOD  | $R^2$ | $A$  |
| Days to heading              | 3     | RM231-C74        | 6.61* | 16.0  | 1.96  | 2     | XNpb39-RM6       | 2.63  | 8.5   | 1.75 |
|                              | 3     | R1927            | 5.13  | 11.0  | 1.61  | 4     | RM252-XNpb331    | 3.35  | 9.5   | 1.90 |
|                              | 4     | XNpb197          | 2.81  | 6.9   | 1.28  | 10    | XNpb37           | 4.01  | 11.9  | −2.23|
|                              | 7     | XNpb333          | 6.07  | 14.2  | 1.86  |        |                  |       |       |      |
| Carbon isotope discrimination($\Delta$) | 2     | RM53             | 3.21  | 7.1   | 0.15  | 1     | XNpb147-RM212    | 4.07  | 9.3   | −0.20|
|                              | 2     | G1327-XNpb227    | 3.12  | 8.0   | 0.16  | 2     | RM53             | 2.67  | 5.90  | 0.16 |
|                              | 6     | RM340            | 3.89  | 8.7   | −0.17 | 2     | G1327-XNpb227    | 3.10  | 8.70  | 0.19 |
|                              | 7     | XNpb379-C213     | 5.25  | 14.3  | 0.22  | 2     | RM240-RM166      | 2.53  | 7.10  | −0.17|
|                              | 11    | N099K            | 4.49  | 10.3  | 0.18  | 6     | RM340            | 4.59  | 10.60 | −0.21|
|                              |        |                  |       |       |       | 7     | C213             | 3.57  | 8.10  | 0.18 |
|                              |        |                  |       |       |       | 11    | N099K            | 4.08  | 10.10 | 0.21 |
| Leaf nitrogen content        | 2     | RM234            | 4.95  | 13.1  | −0.14 | 1     | XNpb147-RM212    | 2.67  | 7.6   | −0.11|
|                              |        |                  |       |       |       | 1     | RM104-XNpb393B   | 4.65  | 14.5  | 0.15 |

* Bold indicates nearest marker of putative QTL.

$^b$ Percent phenotypic variance explained by each QTL.

$^c$ Additive effect of the allele from Milyang 23 compared with Akihikari.

$^*$ Putative QTLs with significant LOD scores on 1000 permutation tests at the 5% level.
Fig. 3. Mapping of QTLs for days-to-heading, carbon isotope discrimination (\(\Delta\)), and leaf nitrogen content at heading stage in 2002 wet season (02DS) and 2003 dry season (02WS) on the rice linkage map. The linkage map consisted of 192 RFLP markers and 81 SSR markers (Fukuta et al., 2004). The rectangular boxes on chromosomes represent the estimated centromere regions (Singh et al., 1996). The numbers, S and L indicate each chromosome, short and long arms, respectively. Markers are located on the left of each chromosome. Triangles and boxes on the right of each chromosome represent LOD peaks of putative QTLs and their one-LOD support intervals (van Ooijen, 1992), respectively. Upward and downward triangles indicate that the Milyang 23 and Akihikari alleles increased each trait, respectively.
for $\Delta$ with different rice populations under irrigated conditions (Ishimaru et al., 2001; Price et al., 2002). Positional coincidence of a QTL across studies can provide support for the validity of the QTL. Of six QTLs detected by Ishimaru et al. (2001), three were on chromosome 2, 6, and 8 where our study identified QTLs for $\Delta$. However, the nearest marker locus to each QTL on these chromosomes in the two studies differed, indicating that these were not common QTLs. Two QTLs were located at the distal end of the long arm on chromosomes 2 and 6 in our study and the study by Price et al. (2002). Although these two QTL appeared to be identical in both studies, the lack of information on marker names in the linkage map by Price et al. (2002) prevents us from comparing the location of QTLs.

One of the most interesting results in the present study was that a wide variation including transgressive segregation was observed for $\Delta$ in the current population (Fig. 2). While the difference in $\Delta$ between Milyang 23 and Akihikari at the heading stage was only 0.28 and 0.43 % in 2002 and 2003, about 3 % variation was transgressively observed among the RILs in both years. The most plausible cause proposed for transgression is accumulation of complementary alleles at multiple loci inherited from the two parents in the progeny (Tanksley, 1993). Four out of seven detected QTLs were those for $\Delta$ that is increased by the Milyang 23 allele, whereas the other three were those for $\Delta$ that is increased by the Akihikari allele. No epistatic effect was confirmed among the seven QTLs for $\Delta$ by the two-way ANOVA for each marker pair (data not shown). These results indicate that a combination of those QTLs may have produced the progenies which are superior or inferior to the parental phenotypes. However, $\Delta$ in RILs was substantially skewed toward lower values. This kind of segregation distortion is often considered due to hybrid weakness (Fukukoa et al., 1998), so that there is a possibility that detected QTLs were associated with it. Fukuta et al. (1998) detected a hybrid weakness, which was governed by a set of two complementary QTLs on chromosome 2 and 11, using the same population in the temperate condition of Japan. These QTLs were localized at the interval of two RFLP markers, XNp199 and XNp639, on chromosome 2, and the other one between two PRFL markers, XNp179 and XNp202, on chromosome 11. Milyang 23 and Akihikari alleles contributed to the hybrid weakness on chromosomes 2 and 11, respectively. The QTL for $\Delta$ on chromosome 11 was detected at the same chromosome region as hybrid weakness, but those on chromosome 2 were different from the QTL for hybrid weakness on chromosome 11. Hybrid weakness was not observed in the tropical condition of IRRI (data not shown). Thus, we could not conclude that the hybrid weakness was associated with the transgressive segregation of $\Delta$ directly. Evaluation of $\Delta$ and its relationship with hybrid weakness using near isogenic lines for detected QTLs will help us to elucidate whether the segregation distortion in $\Delta$ depends on hybrid breakdown or not.

Detection of above-mentioned QTLs, despite the similar $\Delta$ in Milyang 23 and Akihikari, also suggests that $^{13}$C in Milyang 23 may have been discriminated through a photosynthetic process different from that in Akihikari. According to the theory reported by Farquhar et al. (1982), $\Delta$ is related to $\text{Ci}/\text{Ca}$ and determined by the balance between stomatal conductance and carboxylation efficiency in the chloroplasts. This suggests that two parental cultivars may have different magnitudes of stomatal conductance and/or carboxylation efficiency, and that detected QTLs for $\Delta$ may be associated with any of these traits. Hence, we investigated photosynthetically associated traits in the two parents and analyzed the association between $\Delta$ and those traits on the basis of CO$_2$ diffusive model (Farquhar and Sharkey, 1982). $\text{Pn}$ can be expressed from viewpoints of both the supply and demand for CO$_2$ as follows

\[ \text{Pn} = \text{gco}_2 \cdot (\text{Ca}-\text{Ci}) \]  \hspace{1cm} (3)
\[ \text{Pn} = \text{IS} \cdot (\Gamma - \text{IS} \cdot \text{gco}_2) \]  \hspace{1cm} (4)

where $\Gamma$ is the CO$_2$-compensation point. IS governs the carboxylation efficiency of the leaf (Lambers et al., 1998). Eqn 4 is valid as long as $\text{Pn}$ linearly increases with increasing $\text{Ci}$. Since the linear increase in $\text{Pn}$ was observed in Milyang 23 and Akihikari at less than ambient CO$_2$ concentration in this study (Fig. 1), we regarded that Eqn 4 was effective for the two cultivars. Combining Eqs 3 and 4 gives

\[ \text{Gi} = \Gamma + \frac{1}{1 + \frac{\text{IS} \cdot \text{gco}_2}{\text{Ca} - \text{Ci}}} \]  \hspace{1cm} (5)

Eqn 5 means that a higher $\text{gco}_2$ and/or a lower IS will lead to a higher $\text{Gi}$ provided that $\Gamma$ is constant among cultivars. There was no difference in $\Gamma$ between the two cultivars (Table 1). Milyang 23 showed a higher $\text{Gi}/\text{Ca}$ than Akihikari at both reproductive and heading stages. The $\text{gco}_2$ was significantly higher in Milyang 23 than in Akihikari at both stages in contrast to the inconsistent result of IS. Thus, the higher $\text{Gi}/\text{Ca}$ in Milyang 23 may mostly be attributed to the higher $\text{gco}_2$. According to the Eqn 1 proposed by Farquhar et al. (1982), a higher $\text{Gi}/\text{Ca}$ leads to a higher $\Delta$. Therefore, on the basis of this theory, we suggest that the QTLs for $\Delta$ that is increased by the Milyang 23 allele might be related to a higher stomatal conductance.

However, this theory provides no persuasive traits to explain the detected QTLs for $\Delta$ that is increased by the Akihikari allele, because Akihikari showed a lower $\text{Gi}/\text{Ca}$ through a higher IS and/or a lower $\text{gco}_2$. 
It should be noted that although \( \text{Ci}/\text{Ca} \) in Milyang 23 was significantly different from that in Akihikari at the heading stage (0.78 and 0.71, respectively), there was little difference in \( \Delta \) between the two cultivars (21.22 and 21.46\%, respectively). The values of \( \text{Ci}/\text{Ca} \), 0.78 and 0.71, correspond to 23.59 and 21.87 \%e of \( \Delta \) in Eqn 1, where a and b are 4.4 and 29 \%, respectively. Possible explanations for this discrepancy may be as follows. First, Eqn 1 is based on the assumption that discrimination by Rubisco depends on \( \text{Ci} \) even though it can occur at carboxylation sites in the chloroplast. The concentration gradient of \( \text{CO}_2 \) was indeed observed between intercellular airspaces and chloroplasts in rice plants (Sasaki et al., 1996; Hanba et al., 2004). Therefore, the \( \text{CO}_2 \) drop from intercellular air spaces to carboxylation site in Milyang 23 and Akihikari may be different, resulting in a similar \( \Delta \) value in the two cultivars. The \( \text{CO}_2 \) gradient from air to chloroplasts needs to be determined to confirm this hypothesis. Another explanation may be that we compared \( \Delta \) in dry matter to instantaneous gas exchange data. Because \( \Delta \) in the whole leaf sample contained cumulative discrimination during dry matter formation, the short-term gas exchange measurement may not sufficiently reflect the long-term result of \( \Delta \). However, instantaneous and integrated discrimination were found to be closely related in a previous study (Evans et al., 1986), and QTLs for \( \Delta \) even in leaf dry matter were stably detected in our two-year experiments. The major advantage of using \( \Delta \) in the stored sample over the instantaneous result is that a great number of samples can be evaluated, which is more preferable for the breeding program than the gas exchange measurement that is difficult and time-consuming. Near isogenic lines are being developed for targeting QTLs controlling \( \Delta \) to elucidate how the QTLs could influence photosynthesis and its associated traits such as stomatal conductance.

So far, a number of QTLs have been detected for \( \Delta \) in several crops under the field conditions. However, few studies have examined the functions of those QTLs in photosynthetic system. The present study detected several QTLs for \( \Delta \), and clarified significant differences in leaf photosynthetic system between Milyang 23 and Akihikari. Although it cannot be concluded that the detected QTLs can play an important role in the increase of stomatal conductance and \( \text{CO}_2 \) gradient between intercellular airspaces and chloroplasts, current identification of QTLs controlling \( \Delta \) is a step toward improvement of rice leaf photosynthesis.

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**References**

Amani, I., Fischer, R.A. and Reynolds, M.P. 1996. Canopy temperature depression association with yield of irrigated spring wheat cultivars in a hot climate. J. Agron. Crop Sci. 176 : 119-129.

Basten, C.J., Weir, B.S. and Zeng, Z.B. 2002. QTL Cartographer. Version 1.16. A Reference Manual and Tutorial for QTL Mapping. Department of Statistics, North Carolina State University, Raleigh, North Carolina.

Bremner, J.M. and Mulvaney, C.S. 1982. Nitrogen-total. In A.L. Page, R.H. Miller and D.R. Keeney eds., Methods of Soil Analysis, Part 2. American Society of Agronomy, Madison, Wisconsin. 595-624.

Breidel, O., Pot, D., Plomion, C., Rozenberg, P. and Guelhl J.M. 2002. Genetic parameters and QTL analysis of \( \delta^{13} \text{C} \) and ring width in maritime pine. Plant Cell Environ. 25 : 945-953.

Churchill, G.A. and Doerge, R.W. 1994. Empirical threshold values for quantitative trait mapping. Genetics 138 : 963-971.

Condon, A.G., Richards, R.A. and Farquhar, G.D. 1987. Carbon isotope discrimination is positively correlated with grain yield and dry matter production in field-grown wheat. Crop Sci. 27 : 996-1001.

Condon, A.G., Richards, R.A., Rebetzke, G.J. and Farquhar, G.D. 2002. Improving intrinsic water-use efficiency and crop yield. Crop Sci. 42 : 122-151.

Condon, A.G., Richards, R.A., Rebetzke, G.J. and Farquhar, G.D. 2004. Breeding for high water-use efficiency. J. Exp. Bot. 55 : 2447-2460.

Doerge, R.W. and Churchill, G.A. 1996. Permutation tests for multiple loci affecting a quantitative character. Genetics 142 : 285-294.

Earl, H.J. 2002. Stomatal and non-stomatal restrictions to carbon assimilation in soy bean (Glycine max) lines differing in water use efficiency. Environ. Exp. Bot. 48 : 237-246.

Evans, J.R., Sharkey, T.D., Berry, J.A. and Farquhar, G.D. 1986. Carbon isotope discrimination measured concurrently with gas exchange to investigate \( \text{CO}_2 \) diffusion in leaves of higher plants. Aust. J. Plant Physiol. 13 : 281-292.

Farquhar, G.D. and Sharkey, T.D. 1982. Stomatal conductance and photosynthesis. Annu. Rev. Plant Physiol. 33 : 317-345.

Farquhar, G.D., O’Leary, M.H. and Berry, J.A. 1982. On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. Aust. J. Plant Physiol. 9 : 121-137.

Fukuoka, S., Namai, H. and Okuno, K. 1998. RFLP mapping of the genes controlling hybrid breakdown in rice (Oryza sativa L.). Theor. Appl. Genet. 97 : 446-449.

Fukuta, Y., Sato, T., Tamura, T., Yagi. T. 1998. Genetic and breeding analysis using molecular markers 13. Characterization of two complementary recessive genes for hybrid weakness derived from the cross between japonica and indica rice (Oryza sativa L.). Breeding Science vol.48 (Supple. 2) : 106.

Fukuta, Y., Araki, E., Uga, Y., Sasahara, H., Tamura, K., .
Nakamura, I., Fukuyama, T., Yanoria, M.J., Ebron, L., Santos, R.E. and Escueta, D.M. 2004. Development of recombinant inbred lines and chromosome segment substitution lines derived from a wide cross between Indica and Japonica type rice (Oryza sativa L.). In World Rice Research Conference 2004 abstract. 285.

Hanba, Y.T., Shibasaka M., Hayashi, Y., Hayakawa, T., Kasamo, K., Terashima, I. and Katsuahara, M. 2004. Overexpression of the barley aquaporin HvPIP2;1 increases internal CO2 conductance and CO2 assimilation in the leaves of transgenic rice plants. Plant Cell Physiol. 45 : 521-529.

Horie, T., Lubis, I., Takai, T., Ohsumi, A., Kuwasaki, K., Katsura, K. and Nii, A. 2003. Physiological traits associated with high yield potential in rice. In T.W. Mew, D.S. Brar, S. Peng, D. Dawe and B. Hardy eds., Rice Science: Innovations and Impact for Livelihood, IRRI, Los Banos, Philippines. 117-145.

Ishihara, K., Iida, O., Hirasawa, T. and Ogura, T. 1979. Relationship between nitrogen content in leaf blades and photosynthetic rate of rice plants with reference to stomatal aperture and conductance. Jpn. J. Crop Sci. 48 : 543-550.

Ishimaru, K., Yano, M., Aoki, N., Ono, K., Hirose, T., Lin, S.Y., Monna, L., Sasaki, T. and Ohsugi, R. 2001. Toward the mapping of physiological and agronomic characters on a rice function map: QTL analysis and comparison between QTLs and expressed sequence tags. Theor. Appl. Genet. 102 : 793-800.

Kuroda, E. and Kumura, A. 1990. Difference in single leaf photosynthesis between old and new rice varieties. I. Single-leaf photosynthesis and its dependence on stomatal conductance. Jpn. J. Crop Sci. 59 : 283-292.

Lambers, H., Chapin, F.S. and Pons, T.L. 1998. Plant Physiological Ecology. Springer-Verlag, New York. 1-540.

Lin, H.X., Yamamoto, T., Sasaki, T. and Yano, M. 2000. Characterization and detection of epistatic interactions of 3 QTLs, Hd1, Hd2, and Hd3, controlling heading date in rice using nearly isogenic lines. Theor. Appl. Genet. 101 : 1021-1028.

Makino, A. 2003. Rubisco and nitrogen relationship in rice: leaf photosynthesis and plant growth. Soil Sci. Plant Nutr. 49 : 319-327.

Peng, S., Cassman, K.G. and Kropff, M.J. 1995. Relationship between leaf photosynthesis and nitrogen content of field-grown rice in tropics. Crop Sci. 35, 1627-1630.

Price, A.H., Cairns, J.E., Horton, P., Jones, H.G. and Griffiths, H. 2002. Linking drought-resistance mechanisms to drought avoidance in upland rice using a QTL approach: progress and new opportunities to integrate stomatal and mesophyll responses. J. Exp. Bot. 53 : 989-1004.

Samejima, M. 1985. Intraspecific variations of 13C-discrimination in Oryza sativa L. Bull. Nat. Inst. Agrobiol. Resour. 1 : 63-84.

Sasaki, H., Samejima, M. and Ishii, R. 1996. Analysis by δ13C measurement on mechanism of cultivar difference in leaf photosynthesis of rice (Oryza sativa L.). Plant Cell Physiol. 37 : 1161-1166.

Sinclair, T.R. and Horie, T. 1989. Leaf nitrogen, photosynthesis, and crop radiation use efficiency: a review. Crop Sci. 29 : 90-98.

Singh, K., Ishii, T., Parco, A., Huang, N., Brar, D.S. and Khush, G.S. 1996. Centromere mapping and orientation of the molecular linkage map of rice (Oryza sativa L.). P. Natl. Acad. Sci. USA 93 : 6163-6168.

Specht, J.E., Chase, K., Macrander, M., Graef, G.L., Chung, J., Markwell, J.P., Germann, M., Orf, J.H. and Lark, K.G. 2001. Soybean response to water: a QTL analysis of drought tolerance. Crop Sci. 41 : 493-509.

Teulat, B., Merah, O., Sirault, X. and Barries, C., Waugh, R. and This, D. 2002. QTLs for grain carbon isotope discrimination in field-grown barley. Theor. Appl. Genet.106 : 118-126.

Thumma, B.R., Naidu, B.P., Chandra, A., Cameron, D.F., Bahnisch, L.M. and Liu, C. 2001. Identification of causal relationships among traits related to drought resistance in Stylosanthes scabra using QTL analysis. J. Exp. Bot. 52 : 203-214.

Wong, S.C., Cowan, I.R. and Farquhar, G.D. 1979. Stomatal conductance correlates with photosynthetic capacity. Nature 282 : 424-426.

Yano, M. and Sasaki, T. 1997. Genetic and molecular dissection of quantitative traits in rice. Plant Mol. Biol. 35 : 145-153.

* In Japanese with English abstract.

** In Japanese.