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# Analysis by $\delta^{13}C$ Measurement on Mechanism of Cultivar Difference in Leaf Photosynthesis of Rice (*Oryza sativa* L.)

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**Our previous paper showed that cultivar difference of flag leaf photosynthesis (LPS) in rice (*Oryza sativa* L.) was attributed to the difference in mesophyll resistance ($r_m$). In this paper, we tried to divide $r_m$ into $CO_2$ transfer resistance ($r_r$) and $CO_2$ fixation resistance ($r_c$) for further analysis of $r_m$ by means of $\delta^{13}C$ determination of photosynthetic products. In the heading time, $r_r$, and $r_c$ occupied 14, and 58% in the total resistance, respectively, in average through 31 cultivars. This suggests that cultivar difference of LPS is mainly caused by the difference of $r_c$. The percentage ratios of each resistance to the total one were maintained comparatively constant through the senescing process of the flag leaves. Moreover, the $CO_2$ concentration in $CO_2$ fixating site showed almost the same values in different cultivars in a whole life span of a flag leaf. The $r_c$ showed a close correlation with ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) content. Carbonic anhydrase (CA) activity showed no correlation with $r_r$, but showed positive correlation with $r_c$. It was suggested that a mutual regulation system between CA activity and RubisCO activity could exist.**

**Key words:** Carbonic anhydrase (EC 4.2.1.1) — Cultivar difference — $\delta^{13}C$-Mesophyll resistance — *Oryza sativa* L. — RubisCO (EC 4.1.1.39).

Our previous paper (Sasaki and Ishii 1992) showed that cultivar difference of leaf photosynthesis (LPS) in rice (*Oryza sativa* L.) was controlled mainly by the factors relating to mesophyll $CO_2$ resistance ($r_m$). However, $r_m$ involves different kinds of physical and chemical resistances in $CO_2$ diffusion and fixation processes. Isolation of physical resistance from $r_m$ has been so far attempted by several researchers with the measurement of leaf gas exchange and chlorophyll fluorescence (Epron et al. 1995, Loreto et al. 1994), $^{18}O_2$ uptake (Renou et al. 1990), carbon isotope fraction (Evans et al. 1986, von Caemmerer and Evans 1991), light response curve of $CO_2$ exchange (Chartier et al. 1970), and the analysis of the relationship between initial slope of A-Ci response curve and the ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) activity (Evans 1983, Evans and Seemann 1984). Chartier et al. (1970), and Čatský and Tichá (1982) found that the physical resistance occupied as large as 60-80% in the total resistance, and concluded that the physical $CO_2$ transfer step would be the largest limiting one for LPS. On the other hand, it was reported that the physical step resistance was not so large, compared with that in the carboxylation step (Raven and Glidewell 1981, Evans 1983, Evans et al. 1986).

In the present paper, we attempted to divide mesophyll $CO_2$ resistance ($r_m$) to $CO_2$ transfer resistance and fixation resistance by the measurement of $\delta^{13}C$ values of the current photosynthetic products in the leaves of rice plants, to identify the most possible process of $CO_2$ influx which contributes to the cultivar difference of LPS.

It is considered that the $CO_2$ transfer process from stomatal cavity to $CO_2$ fixation site is closely associated with carbonic anhydrase (CA) activity (Makino et al. 1992, Price et al. 1994), the area ratio of mesophyll cell surface to leaf surface (Nobel et al. 1975), or the ratio of chloroplast surface area to intercellular air space volume (von Caemmerer and Evans 1991, Kariya and Tsunoda 1980). It is also considered that $CO_2$ fixating process in the chloroplast is mainly related with the activity and/or content of RubisCO (Evans 1983, Evans and Seemann 1984).

The activities of CA and RubisCO were, therefore, determined in the cultivars to elucidate the mechanism of cultivar differences in $CO_2$ transfer and fixation resistances.

Abbreviations: CA, carbonic anhydrase; $[CO_2]_{atm}$, $CO_2$ concentration in the atmospheric air; $[CO_2]_{cht}$, $CO_2$ concentration at the $CO_2$ fixating site in the chloroplast; $[CO_2]_{stc}$, $CO_2$ concentration in the stomatal cavity; $\Delta$, carbon isotope discrimination by the plant; $\delta_a$, relative concentration of $^{13}C$ in the atmospheric air; $\delta_p$, relative concentration of $^{13}C$ in the photosynthetic products; DTT, dithiothreitol; $\delta^{13}C$, relative concentration of $^{13}C$ in total carbon atoms; LPS, leaf photosynthesis; PPFD, photosynthetic photon flux density; PVP, polyvinyl pyrrolidone; $r_c$, $CO_2$ fixation resistance; $r_m$, mesophyll $CO_2$ resistance; $r_r$, $CO_2$ transfer resistance; $r_s$, stomatal $CO_2$ resistance; $r_t$, total resistance; RubisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase.

## Materials and Methods

*Cultivation of plant materials*—The seeds of thirty one rice cultivars were sown on the semi-flooded rice nursery bed on 21st April in 1988, and the seedlings were transplanted on 30th May to the paddy field. The cultivation methods and the experimental plots were the same as described in our previous paper (Sasaki and Ishii 1992).

*Measurement of gas exchange rate*—Leaf photosynthesis

(LPS) and transpiration were simultaneously measured on the main stem flag leaves from three plants of each cultivar, under the irradiance above 1,450 $\mu$mol $m^{-2}$ $s^{-1}$ PPFD in the field according to the method already reported (Sasaki and Ishii 1992). The measurements were conducted for each cultivar in 0, 2, and 4 weeks after heading which corresponded to heading, grain filling, and grain maturing time, respectively.

*Extraction of soluble sugars*—Fifteen flag leaves of each cultivar were covered with aluminium foil at 1200h on the precedent day to LPS measurement, to make the leaves starved for the photosynthetic products. On the morning on the day of LPS measurement, the flag leaves were exposed to the sunlight from 0900h to 1200h, and then, they were cut off from the plant and put in liquid nitrogen. They were stored in a deep freezer at −80°C until the determination of soluble sugars. The soluble sugars were extracted following to the modified method of Brugnoli et al. (1988). The frozen leaves were submerged in 80% (v/v) ethanol of 80°C for 6 hours. After the ethanol soluble fraction was evaporated at 40°C, the residue was suspended in 50 ml chloroform and same amount of water. The water soluble fraction was passed through sequentially two columns filled with Dowex-50 ($H^+$ form) and Dowex-1 ($OH^-$ form), to remove the amino acids and other organic acids. The neutral fraction obtained was freeze-dried, and served for the determination of $\delta^{13}C$ value. It was checked whether carbon discrimination occurred in the process of sucrose extraction, by measuring $\delta^{13}C$ of authentic sucrose which was subjected to the same procedure as mentioned above.

*$\delta^{13}C$ determination*—The determination of $\delta^{13}C$ of soluble sugars was made according to the method by Samejima (1985), with a mass spectrometer (Finnigan MAT, San Jose, U.S.A.). Carbon isotope discrimination by the plant ($\Delta$) can be expressed by the following equation (Hubick et al. 1986).

$$\Delta=\frac{\delta_a-\delta_p}{1+\delta_p} \qquad \text{Eq. 1}$$

where $\delta_a$, and $\delta_p$ are the relative concentration of $^{13}C$ in the atmospheric air, and in the photosynthetic products, respectively. The $\delta_a$ was fixed as $-8.0\times10^{-3}$ from the measurement for the air in Tokyo (Samejima 1985), and $\delta_p$ was determined by the mass-spectrometric method with soluble sugars extracted from the flag leaves. The $\Delta$ obtained was inserted to the place of $\Delta$ in Eq. 2, which was the modified equation of Farquhar and Richards (1984).

$$\Delta\times10^3=4.4\times\frac{[CO_2]_{atm}-[CO_2]_{stc}}{[CO_2]_{atm}}+(1.1+0.7)\times\frac{[CO_2]_{stc}-[CO_2]_{cht}}{[CO_2]_{atm}}+29\times\frac{[CO_2]_{cht}}{[CO_2]_{atm}} \qquad \text{Eq. 2}$$

where, 4.4 is the discrimination coefficient in the $CO_2$ diffusion process through stomata in $C_3$ plants (Farquhar and Richards 1984), 1.1 is that in the $CO_2$ dissolution process of $CO_2$ into the water at 25°C (Vogel et al. 1970), 0.7 is that in the $CO_2$ diffusion process in the liquid phase at 25°C (O'Leary 1984), and 29 is that in the $CO_2$ carboxylation process by ribulose-1,5-bisphosphate carboxylase (Evans et al. 1986). The carbon discrimination occurred also in the processes of dark respiration and photorespiration. However, we assumed these factors to be zero, because according to the several papers, its extent is almost negligible (Evans et al. 1986, Farquhar et al. 1982). The $[CO_2]_{atm}$, $[CO_2]_{stc}$, and $[CO_2]_{cht}$ are the concentrations of $CO_2$ in the atmospheric air, in the stomatal cavity, and at the $CO_2$ fixating site of RubisCO in the chloroplast, respectively. The $[CO_2]_{atm}$ was maintained constant as 340 $\mu$l $liter^{-1}$ in this experiment, and $[CO_2]_{stc}$ was obtained from the measurement of LPS and transpiration as previously reported (Sasaki and Ishii 1992). The $[CO_2]_{cht}$, therefore, can be obtained from Eq. 2 when the value of $\Delta$ determined from Eq. 1 is put in the place of $\Delta$ in Eq. 2.

*Calculation of stomatal, transfer and fixation resistances*—The determination of stomatal resistance ($r_s$) was made according to the method by Sasaki and Ishii (1992). If $[CO_2]_{cht}$ is obtained from Eq. 2, the $CO_2$ transfer resistance ($r_r$) from stomatal cavity to the $CO_2$ fixating site and fixation resistance ($r_c$) can be obtained by the following equation.

$$r_r=\frac{[CO_2]_{stc}-[CO_2]_{cht}}{LPS} \qquad \text{Eq. 3}$$

$$r_c=\frac{[CO_2]_{cht}}{LPS} \qquad \text{Eq.4}$$

*Determination of RubisCO content*—Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) (EC 4.1.1.39) content was determined on the main stem flag leaves at heading, grain filling and grain maturing stages. The leaves were sampled immediately after the LPS measurement, and then stored at −80°C in the freezer until measurement. The leaves were ground with quartz sand in the buffer solution (pH 7.9) containing 50 mM Tris-Hcl, 10 mM $MgCl_2$, 0.5 mM EDTA-Na, 5 mM DTT and 0.2% (w/v) PVP. After the homogenate was centrifuged at 12,000×*g* for 30 min, the supernatant was served for the determination of RubisCO content. The RubisCO was assayed by the single radial immunodiffusion method with the gel containing 1.2% agarose LE, 100 mM Tris, 25 mM HCl, 0.1% (w/v) $NaN_3$ and anti RubisCO serum (Fahey and Mckelvey 1965).

*Determination of carbonic anhydrase activity*—Carbonic anhydrase (CA) (EC 4.2.1.1) activity was measured on the main stem flag leaves at heading, grain filling and grain maturing stages. The detached leaves were ground with buffer solution (pH 8.3) containing 50 mM barbital-$H_2SO_4$, 5 mM DTT and 0.2% (w/v) PVP. The ground soup was centrifuged at 12,000×*g* for 2 min, and the supernatant was served for the determination of CA activity. The CA activity was determined according to the method by Tsuzuki et al. (1985). In a preliminary experiment, the inhibition of CA activity by the added DTT was checked, and no inhibition was observed.

## Results

*Soluble sugar content in the leaves for $\delta^{13}C$ measurement*—In order to determine $\delta^{13}C$ for the current photosynthetic products, the leaves were starved for accumulated soluble sugars by covering the leaves with aluminium foil as described in Materials and Methods. The soluble sugar content decreased from 32.37 mg (g FW)$^{-1}$ to 5.09 mg (g FW)$^{-1}$ in 6 hours after the leaves were subjected to darkness, reaching 2.68 mg (g FW)$^{-1}$ in 21 hours of the continued dark period (Fig. 1). However, if the leaves were exposed to sunlight, the soluble sugar content recovered to 33.58 mg (g FW)$^{-1}$ only in 3 hours of exposure to the light. This indicates that the accumulated soluble sugars can be replaced with the newly formed ones by 3 hours of reillumination after 21 hours of darkness. It was verified, therefore, that the measurement of $\delta^{13}C$ in the reilluminated leaves after

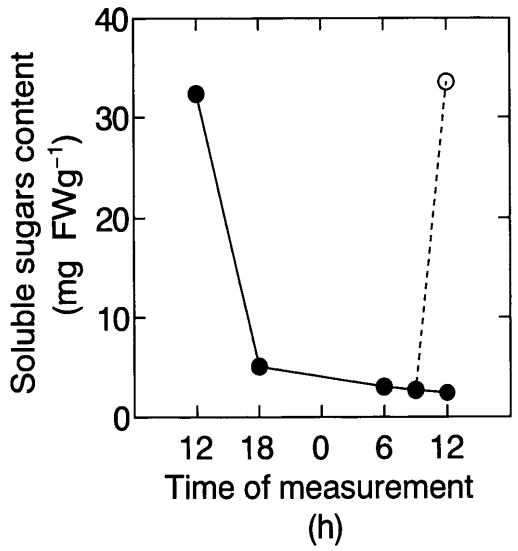


**Fig. 1** Change of soluble sugars content in the leaves after the initiation of darkness. Measurement was conducted on the upper most fully expanded leaves. The values are means of 10 measurements. Darkness was initiated at 1200h. The leaves were exposed to full sunlight for 3 h (0900h–1200h) before initiation of darkness. ●, the plants in darkness; ○, the plants re-exposed to the sunlight at 0900h of the next day. Content of soluble sugars was determined enzymatically according to the method by Nakamura and Yuki (1992).

21 hours of starvation treatment, was done on the current photosynthetic products.

*Mean LPS, Δ and their related parameters through all cultivars*—Mean values of LPS, Δ, and their related parameters through 31 cultivars in 3 different senescing stages of the flag leaves are shown in Table 1. Mean LPS decreased with leaf senescence, reaching about half of the mean value in the heading time. It should be noticed that though the absolute values of $r_s$, $r_r$ and $r_c$ increased with progress of senescence, their relative values to total resistance ($r_t$) were maintained almost constant. This suggests that a mutual regulation mechanism in these resistances exists. Moreover, this regulation mechanism produce an unchanged $[CO_2]_{cht}$ through these different stages of leaf senescence. It should be also noticed that the relative $r_r$ was so low as 14% in the heading time, and 18% in the grain maturing time. This means that the extent of contribution of $r_r$ to LPS determination is not so great as we considered from the past papers. (Čatský and Tichá 1982, Chartier et al. 1970). On the other hand, contrary to $r_r$, $r_c$ occupied so much as 58, and 60% in $r_t$, in the heading, and in the grain filling stage, respectively. This indicates that the contribution of $r_c$ is great to the determination of LPS.

*Cultivar difference of LPS and its determination mechanism*—The LPS showed a comparatively large variation as from 16.8 to 23.3 $\mu$mol m$^{-2}$ s$^{-1}$ in the heading time, and from 11.8 to 18.8 $\mu$mol m$^{-2}$ s$^{-1}$ in the grain filling time. To elucidate the determination mechanisms of cultivar difference in LPS, the relationships between LPS and the reciprocals of $r_s$, $r_r$, and $r_c$ which conceptually correspond to the $CO_2$ conductance in each step were examined (Fig. 2). Though significant correlation was not necessarily observed between LPS and $1/r_s$ or $1/r_r$, high significant correlation was consistently seen between LPS and $1/r_c$ in any leaf senescing stage. This indicates that the cultivar difference in LPS is attributed to the factors relating to $CO_2$ fixating step rather than those relating to $CO_2$ stomatal diffusion or $CO_2$ transfer step.

To elucidate the biochemical mechanism of cultivar difference in $r_c$, the RubisCO content, which is supposed to be closely related with $CO_2$ fixating step, was measured in

**Table 1** The mean values of flag leaf photosynthesis (LPS), carbon isotope discrimination (Δ), and $CO_2$ stomatal ($r_s$), transfer ($r_r$) and fixation ($r_c$) resistance, and $CO_2$ concentration in the stomatal cavity ($[CO_2]_{stc}$) and at the fixating site ($[CO_2]_{cht}$) in different stage of flag leaf senescence

| Parameter | Stage of flag leaf | | |
|---|---|---|---|
| | Heading | Grain filling | Grain maturing |
| LPS ($\mu$mol m$^{-2}$ s$^{-1}$) | 20.3 ± 1.6 | 16.0 ± 1.5 | 11.6 ± 1.6 |
| Δ ($10^{-3}$) | 18.25± 0.44 | 18.45± 0.63 | 18.61± 0.66 |
| $r_s$ (mol$^{-1}$ m$^2$ s) | 4.2 ± 0.6 (28±3) | 4.5 ± 0.6 (23±4) | 6.1 ± 1.2 (22±4) |
| $r_r$ (mol$^{-1}$ m$^2$ s) | 2.1 ± 0.5 (14±3) | 3.5 ± 0.9 (18±4) | 4.9 ± 1.3 (18±2) |
| $r_c$ (mol$^{-1}$ m$^2$ s) | 8.7 ± 0.8 (58±2) | 11.6 ± 1.3 (59±2) | 16.3 ± 3.1 (60±3) |
| $[CO_2]_{stc}$ ($\mu$l liter$^{-1}$) | 245 ±10 | 261 ±13 | 263 ±12 |
| $[CO_2]_{cht}$ ($\mu$l liter$^{-1}$) | 197 ± 6 | 201 ± 7 | 203 ± 9 |

Values are expressed as Mean±SE through 31 cultivars. Figures in the parentheses are percentage ratio of each resistance to total resistance.

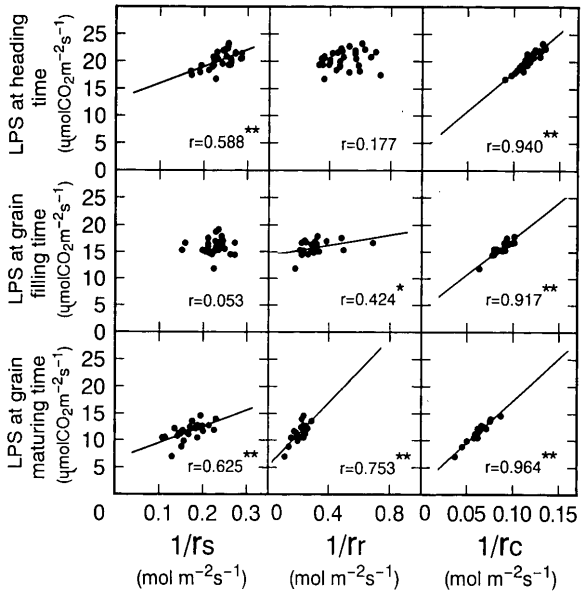


**Fig. 2** Relationships between flag leaf photosynthesis (LPS) and the reciprocal of $r_s$, $r_r$, and $r_c$ in heading, grain filling, and grain maturing time. Lines, and figures represent regression lines, and correlation coefficients, respectively. *, **, significant at the 0.05, and 0.01 probability levels, respectively.

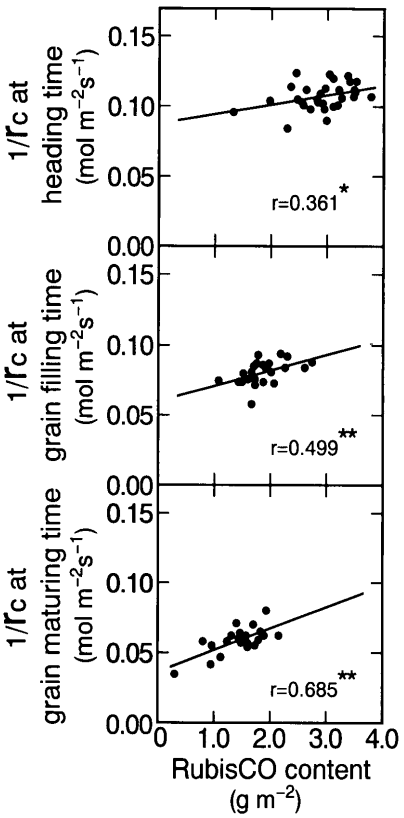


**Fig. 3** Relationships between RubisCO content and $1/r_c$ in heading, grain filling, and grain maturing time. Lines, and figures represent regression lines, and correlation coefficients, respectively. *, **, significant at the 0.05, and 0.01 probability level, respectively.

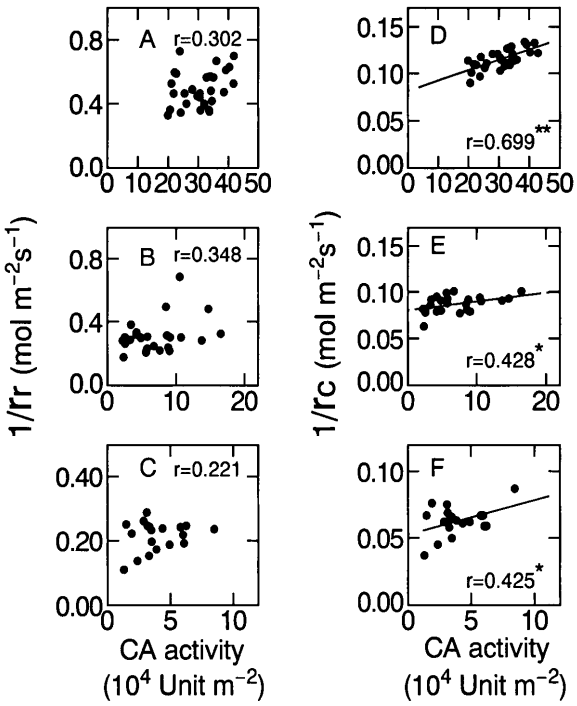


**Fig. 4** Relationships between carbonic anhydrase (CA) activity and $1/r_r$ and $1/r_c$ in heading (A, D), grain filling (B, E), and grain maturing time (C, F). Lines, and figures represent regression lines, and correlation coefficients, respectively. *, **, significant at the 0.05, and 0.01 probability level, respectively.

each cultivar at different leaf senescing stage. Figure 3 shows the correlation between RubisCO content and $1/r_c$ at the different leaf senescing times, and significant correlation was obtained at any leaf senescing time. This is indicating that cultivar difference in LPS was determined to large extent by RubisCO content.

To investigate the relationship of enzymatic factors with $CO_2$ transfer step, the activity of carbonic anhydrase (CA), which has been considered as an enzyme closely relating to $CO_2$ transfer step, was measured (Fig. 4). The activity of CA ranged from 19.9 to $41.9 \times 10^4$ Unit $m^{-2}$ in the heading time and decreased rapidly with progress of leaf senescence. Though the activity of CA was expected to correlate with $1/r_r$, significant correlation was not actually observed in any senescing time. However, significant correlation was seen between CA activity and $1/r_c$ in all senescing stages of the leaf.

## Discussion

The first objective of this paper was to find what step in the photosynthetic $CO_2$ assimilation process was the main cause of cultivar difference in LPS of rice. We attempted to divide mesophyll $CO_2$ resistance ($r_m$) into $CO_2$ transfer resistance ($r_r$) and carboxylation resistance ($r_c$) by mass

spectrometric measurement of $\delta^{13}C$ in the current photosynthetic products.

Estimation of $r_r$ could contribute to the evaluation of $r_r$ to the determination of LPS. von Caemmerer and Evans (1991) found that an average $r_r$ of wheat cultivars was 2.4 $mol^{-1}\ m^2\ s$, and Loreto et al. (1994) also found that $r_r$ of young wheat leaf ranged from 1.7 to 2.9 $mol^{-1}\ m^2\ s$. For rice, on the other hand, von Caemmerer and Evans (1991) reported that $r_r$ were 2.0 and 2.6 $mol^{-1}\ m^2\ s$. The $r_r$ obtained in the present paper ranged from 1.4 to 3.1 $mol^{-1}\ m^2\ s$ at heading time, and they were comparable to the values mentioned above. Mean percentage ratio of $r_r$ to $r_t$ through 31 cultivars, was as large as 15% in any senescing stage of the flag leaf, while that of $r_s$ to $r_t$ ranged from 20 to 30%. This suggests that the $CO_2$ transfer process does not play so important role as the limiting factor of LPS compared with $CO_2$ fixating process or diffusion process at stomata. However, $r_r$ showed such a large cultivar difference as 25% by coefficient of variation, which the coefficient of variance of $r_s$, and $r_c$ was 16%, and 13%, respectively. Therefore, it could be considered that the cultivar difference of $r_r$ contributes to the difference of LPS to more extent than predicted from the magnitude of ratio of $r_r$ to $r_t$.

It was another objective of this paper to examine the change of $r_r$ with progress of leaf senescence. Loreto et al. (1994) reported for wheat that $r_r$ increased from 2.2 to 16.7 $mol^{-1}\ m^2\ s$ during the grain maturation period. We found that the values of $r_r$ ranged from 1.5 to 5.7 $mol^{-1}\ m^2\ s$ among the cultivars at the grain filling time, and from 3.5 to 9.1 $mol^{-1}\ m^2\ s$ at the grain maturing time. Though the $r_r$ increased with progress of leaf senescence, the ratio of $(r_s + r_r)$ to $r_c$ was maintained constant through the whole period of leaf senescence (Table 1). This led to such a constant $[CO_2]_{cht}$ as about 200 $\mu l\ liter^{-1}$ through whole life of a flag leaf, as observed in the present study. Furthermore, variation of this ratio among the cultivars were small in any of the heading, grain filling, and grain maturing times. These indicate that the resistance from stomata to $CO_2$ fixating site, might be changing in close association with that in the $CO_2$ fixation process.

In this paper, CA activity was measured with all cultivars in different times of leaf senescence, to examine the relationship between CA activity and $r_r$, because CA can be considered to be relating with the resistance in the $CO_2$ transfer process (Makino et al. 1992, Price et al. 1994). We found, however, that a positive correlation was found between CA activity and $CO_2$ fixation conductance ($1/r_c$), although we failed in finding a positive correlation between CA activity and transfer conductance ($1/r_r$) (Fig. 4). This suggests that the activity of carbonic anhydrase, most of which exists in the chloroplasts (Tsuzuki et al. 1985), changes in association with RubisCO activity in the chloroplast. It was reported by Hudson et al. (1992) that CA activity changed in association with RubisCO activity in transgenic tobacco introduced with antisense against RubisCO mRNA. It was also reported that CA : RubisCO ratio was maintained almost constant in spite of difference in amount of RubisCO and CA among cultivars and among leaf nitrogen contents (Majeau and Coleman 1994, Makino et al. 1992). Considering these experimental results, it could be speculated that a kind of mutual regulation system between CA and RubisCO exists in the chloroplast.

Moreover, the present paper indicated that CA activity showed no correlation with $r_r$. It would be considered that the magnitude of $r_r$ due to the resistance between the stomatal cavity and $CO_2$ fixation site with the anatomical factors of the mesophyll cells, such as mesophyll cell surface area to the leaf area ($A_{mes}/A$) (Evans and Seemann 1984), chloroplast surface area adjacent to plasma membranes (von Caemmerer and Evans 1991), relative ratio of air space inside a leaf (Loreto et al. 1992). However, since we did not examine these parameters, the commitment of these to the transfer process are not obvious.

This paper indicated that the cultivar difference of LPS was caused mainly by the $CO_2$ fixating process which was closely associated with related to the content of RubisCO, and small extent by the $CO_2$ transfer process. This paper also suggested that a mutual regulation system between CA activity and RubisCO activity could exist.

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