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Variations in Leaf Gas Exchange Traits of *Saccharum* Including Feral Sugarcane, *Saccharum spontaneum* L.

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Abstract : The effects of photosynthetic photon flux density (PPFD) and temperature on leaf gas exchange rates of ten lines of *Saccharum spontaneum* L. collected from the tropics to temperate regions in North-West Pacific zones, *S. sinense* Roxb. cv. Yomitanzan, native sugarcane in South-West Islands, and *Saccharum* hybrid cv. NCo310 were investigated. The relationship of gas exchange traits and rates was examined. Significant line- and species-differences ($P<0.05$) were found in the carbon dioxide exchange rate (CER) at high PPFD (1700 μM photon $m^{-2}s^{-1}$), leaf stomatal conductance at low PPFD (180 μM photon $m^{-2}s^{-1}$), contents of total nitrogen, soluble protein, and chlorophyll, PEP carboxylase and malic enzyme activity, and specific leaf area (SLA). CER at high PPFD ($P<0.05$) of Tainan, *S. spontaneum* was significantly higher than that of *Saccharum* hybrid cv. NCo 310, while CERs of JW2, JW32, and JW41 of *S. spontaneum* were significantly higher than that of *S. sinense* Roxb. cv. Yomitanzan. CER of Tainan exhibited the highest value in a range of 20 to 35°C. Consequently, this suggests that *S. spontaneum* such as Tainan, is a useful germplasm source in sugarcane breeding. CER of *Saccharum* correlated significantly with leaf stomatal conductance ($r=0.496$, $P<0.01$), the malic enzyme activity of Mn type ($r=0.838$, $P<0.01$) and Mg type ($r=0.547$, $P<0.01$), and the chlorophyll content ($r=0.466$, $P<0.01$).
Key words : CO_2 exchange rate, Feral sugarcane, Light intensity, Malic enzyme, *Saccharum spontaneum* L., Stomatal conductance, Temperature response.

野生サトウキビ（*Saccharum spontaneum* L.）を含む数種サトウキビ属における葉身のガス交換特性の変異

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要　旨：北西太平洋地域の熱帯から温帯にかけて採集された野生サトウキビ *Saccharum spontaneum* の10系統，種間交雑種（*Saccharum* hybrid）NCo 310，南西諸島の在来種 *S. sinense* cv. Yomitanzan を用いて，個葉のガス交換速度の光強度と温度に対する反応を調査した．ガス交換速度に関与する要因として，気孔伝導度（Gs），全窒素含量（N），可溶性タンパク質含量（SLP），ホスホエノールピルビン酸カルボキシラーゼ（PEPC），NADP-リンゴ酸酵素（ME），フラクション1タンパク質含量（F1P），クロロフィル含量（CHL），比葉面積（SLA）を測定し，ガス交換速度との関連を検討した．強光下（1700 μmol/m^2/s）での CO_2 交換速度（CER_{1700}），弱光下（180 μmol/m^2/s）での気孔伝導度（Gs_{180}），N, SLP, PEPC, ME, F1P, CHL, SLA において，系統・種の間に有意な変異（$P<0.05$）が認められた．Tainan は，20°C から 35°C にわたって供試した *Saccharum* 属のなかで最も高い CO_2 交換速度を示した．つまり，*S. spontaneum* 系統 Tainan はサトウキビの多収性育種素材として優れた特性を有していることが明らかになった．供試した *Saccharum* 属における強光下での CER_{1700} は，気孔（Gs；$r=0.496$，$P<0.01$）及びマンガン型 ME（MEMN，$r=0.838$，$P<0.01$），マグネシュウム型 ME（MEMG，$r=0.547$，$P<0.01$），クロロフィル含量（CHL，$r=0.466$，$P<0.01$）と高い相関を示した．

キーワード：温度反応，ガス交換速度，気孔伝導度，光強度，*Saccharum spontaneum*，野生サトウキビ，リンゴ酸酵素．

Inter-specific hybridization can be easily made in *Saccharum*. *Saccharum spontaneum* played an important role in the introduction of genes resistant to sereh disease[1]. In recent years, feral sugarcane, such as *S. spontaneum*, has gained attention as a germplasm resource with favorable traits, such as tillering capacity, disease resistance and drought tolerance[5,6,7,8,14].

Irvive[2] showed that the photosynthetic rate of *Saccharum* hybrids was intermediate between the parents' rates, and suggested that the

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evaluation of CO_2 exchange rate (CER) was useful in breeding for economic cultivars. Although there have been several studies on morphological traits and sugar content of *S. spontaneum*[5,6,7,14], few studies have investigated photosynthesis of *S. spontaneum*[5]. Nose et al.[13] studied the traits related to leaf gas exchange rate in 33 lines of *S. spontaneum* collected from the South-West Islands, Japan, and found significant differences in those traits. In principal component analysis of the study, *S. spontaneum* was grouped into native islands on the component characterized by CER and transpiration rate at high light intensity and soluble protein content. In addition, as native islands changed from the south to the north, that is, from the subtropics to the temperature zone, the score of the component changed from negative to positive. Sugiyama and Boku[15], Sugiyama et al.[16], Sugiyama and Yamazaki[17], and McWilliams and Feral[4] observed that temperature differences of germplasm origin and breeding place in *Zea mays* cultivars correlated with genetic variation in temperature response of C_4 photosynthesis. Thus it is expected that the temperature factor of the place of origin influences the properties of leaf gas exchange rate in *Succharum* also.

S. spontaneum occurs widely from tropics to temperate regions. The present experiments were carried out to examine the traits of leaf gas exchange of *Saccharum* species, and to identify breeding parents providing high yielding ability on the aspect of photosynthesis, using ten lines of *S. spontaneum*, collected from the tropical and temperate regions of North-Pacific zones, *Saccharum* hybrid cv. NCo310 as a representative of the economic sugarcane cultivar, and *S. sinense* Roxb. cv. Yomitanzan, the native cultivar cultivated for a long time in Far-East Asia, including the South-West Islands and China.

Materials and Methods

The experimental materials are shown with the places of their origin in Table 1. Plant materials of *S. spontaneum* L. and *S. sinense* Roxb. cv. Yomitanzan were obtained from the collections of the Okinawa Prefectural Agricultural Experimental Station. After stalk cuttings were sterilized for one night in 0.05% solution fo "Benleet-T" (Takeda Chem. Co. Ltd.), they were planted in a sand bed on April 17th, and incubated at 35°C. Uniformly germinated materials were transplanted to 1/2000 a Wagner pots filled with coral limestone soil (brown red soil), and grown in a glasshouse at the University of the Ryukyus. As basal dressing, compost containing $N : P_2O_5 : K_2O = 0.54 : 0.12 : 0.42$ (fresh weight %) was applied at the rate of 1 kg/pot, and a compound fertilizer ($N : P_2O_5 : K_2O = 0.5 : 0.344 : 0.438$ gram per pot) was applied on July 5th. The pots were irrigated twice a day.

The gas exchange rate of leaves was measured according to the method described in previous papers[9,11,13]. The responses of gas exchange rate to light intensities were examined for the fully expanded top leaves during the period from July 18th to August 3rd, which corresponded to 82 to 98 days after planting. Responses of gas exchange rate to temperature were examined on the fully expanded top leaves during the period September 3rd to September 20th. Five to six plants of each line and species were used in each experiment. In the light response experiment, light intensity (photosynthetic photon flux density, PPFD) was altered from 1716 to 182 μM $m^{-2}s^{-1}$, leaf temperature was 30.0±0.3°C, and vapor pressure difference between the leaf and air (VPD) was 1.97±0.17 kPa. In the temperature response experiment, leaf temperature was changed from 40.5 to 15.3°C at a PPFD of 990 μM $m^{-2}s^{-1}$ and the VPD was changed from 0.47 to 5.05 kPa with increasing temperature.

Methods of extraction and determination for phosphoenolpyruvate carboxylase (PEP-Case), ribulose-1, 5-diphosphate carboxylase (faction 1 protein, F1P), soluble protein, and total nitrogen were the same as described in previous papers[9,11,13]. NADP malic enzyme was extracted in an iced mortar with 12 m*l* of the extracting solution, which was prepared with 50 mM HEPES-KOH pH 7.5, 0.2 mM EDTA, 2.5 mM $MgCl_2$, 2 mM DTT, 30 mg Polyclar AT, and 1 g sea sand. After being centrifuged twice for 10 min. at 10,000 rpm, the supernatant was obtained for enzyme activity measurements. The activity of NADP malic enzyme was detected spectrophotometrically as an increase in the rate of NADPH in the reaction mixture : 25 mM Tris-HCl pH 8.0, 5 mM malic acid, 5 mM DTT, 1 mM NADP and 1 mM $MgCl_2$ or 1 mM

Table 1. Materials used and their origin.

Species	Name and origin
Saccharum Hybrid	NCo310
S. sinense Roxb.	Yomitanzan (Kume Island), Yomitanzan (ROC)
S. spontaneum L.	JW2(Ishigaki I.), JW16(Toku I.), JW25(Toku I.), JW32(Kikai I.), LW35(Hawaii), JW41(ROC), JW43(Java), JW45(Ponape), US56-15-2(Thailand), Tainan(ROC)

Name in parentheses of *S. sinense* Roxb. and *S. spontaneum* L. indicates cultivation area and/or the place collected. ROC, US and JW indicates Republic of China, United States of America, and Japanese Wild, respectively.

$MnCl_2$. Chlorophyll was extracted with 99% ethanol solution for 24 hours in the dark and measured with a spectrophotometer. Specific leaf area (SLA) was determined using all of the leaf blades, which were then used for measurement of light intensity response of CER, excluding mid-ribs. Internal traits of leaves excepting total nitrogen content were analyzed after measurement of CER.

Results

Significant differences in the traits of gas exchange of sugarcane leaves were observed within the lines and the species (Table 2). Since light intensity response of CER was characterized by rates at high and low light intensities, CERs at 1716 ± 83 μM $m^{-2}s^{-1}$ (high light intensity) and 182 ± 17 μM $m^{-2}s^{-1}$ (low light intensity) were compared within lines and species. There were no significant differences in CER at low light intensity, transpiration rates at both light intensities, and stomatal conductance at high light intensity. CER at high light intensity, internal factors concerning leaf gas exchange rate and specific leaf area (SLA) showed a significant difference ($P<0.05$) within lines and species. Most CER at high light intensity revealed the typical high rates of C_4-type photosynthesis. Especially, Tainan, *S. spontaneum* possessed the highest CER, while *S. sinense* Roxb. cv. Yomitanzan showed the lowest CER. The ratio of the CER of Tainan to that of *S. sinense* Roxb. cv. Yomitanzan was about 1.5. NCo310 showed an intermediate value of CER.

The temperature response of CER in *Saccharum* is shown in Fig. 1. Significant differences ($P<0.01$) were obsered in the temperature levels, lines and the interactions of temperature treatments and lines. Based on the temperature responses of CER, the materials used were divided into two types. The first group, which exhibited an optimum temperature from 30 to 35°C for higher CER, included most *Saccharum* used, such as NCo310. The second group revealed a wide range of optimum temperatures from 25 to 40°C. US56-15-2 and JW2 belonged to this group, but their CERs were lower than those of the first group. The optimum temperatures showed no relationships to the temperature conditions in the origin of each line and species. In the temperature response, NCo310 possessed a higher CER under leaf temperatures from 15 to 40°C, while Tainan, which provided the highest CER in Table 2, also showed a higher CER at the same range of leaf temperature.

The correlation matrix of gas exchange traits is shown in Table 3. CER at high light intensity was significantly correlated with activity and/or content of NADP malic enzyme of Mn type, chlorophyll, fraction 1 protein (ribulose-1, 5-bisphosphate carboxylase), and soluble protein. There was also a significant correlation between CER and leaf stomatal conductance at high light intensity. CER at high light intensity showed no correlation with total nitrogen content or specific leaf area (SLA). CER at high light intensity is significantly correlated with CER at low light intensity in *Saccharum*.

CER under low light intensity showed a significant and positive correlation with leaf stomatal conductance at low light intensity or malic enzyme activity of Mn type. The correlation coefficient, however, was too low to show any significant correlation with the other traits related to gas exchange rates.

The stomatal conductance of the sugarcane leaf significantly correlated to CER in both

Table 2. Variations in gas exchange rate and related traits of single leaf in *Saccharum*.

Sample	CER_{1700}	CER_{180}	T_{1700}	T_{180}	Gs_{1700}	Gs_{180}	N	SLP	FIP	PEPC	MEMG	MEMN	CHL	SLA
NCo310	36.5	7.21	4.46	1.12	407.8	98.8	1.98	2.53	841	86	120	88	424	167
YT/ROC	29.5	6.50	4.45	0.81	393.4	79.4	2.16	2.27	579	15	107	83	370	222
YT/Kume	30.5	7.81	4.59	1.16	407.3	131.0	2.08	2.37	811	65	129	94	403	206
JW2	37.3	7.69	4.65	0.90	359.7	66.4	2.11	2.51	653	107	119	90	364	192
JW16	35.8	6.25	4.66	0.68	372.0	49.5	1.75	2.42	557	138	117	90	520	159
JW25	33.4	7.28	4.17	0.50	382.0	37.6	1.82	1.91	459	33	94	85	388	183
JW32	40.2	7.53	5.51	0.90	339.9	67.2	1.85	2.39	750	64	130	108	407	164
JW35	36.0	7.62	5.36	1.15	475.2	113.7	2.00	2.14	647	102	115	90	312	229
JW41	37.8	8.05	4.12	0.67	282.6	47.2	2.03	2.29	624	23	92	74	323	211
JW43	33.4	7.38	4.19	0.98	285.0	68.9	1.64	2.30	536	16	107	88	447	173
JW45	34.7	7.35	4.26	1.30	303.6	83.2	1.80	1.93	633	34	113	86	407	211
US56-2	36.4	7.28	4.79	0.64	387.4	505.5	2.38	2.69	856	109	93	67	372	224
Tainan	48.2	8.18	5.52	0.93	416.3	94.8	2.06	2.42	956	35	140	120	492	192
F value	4.68	0.62	1.26	1.38	1.51	3.0	10.7	2.5	5.15	2.6	3.2	4.6	2.3	12
LSD	0.05	n.s.	n.s.	n.s.	n.s.	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
s-LDS	6.6	—	—	—	—	46.8	0.19	0.43	200	77	26	49	117	21

CER_{1700}, Carbon exchange rate (CER) under high photosynthetic photon flux density (1700 μM m^{-2} s^{-1}, HPPFD), μM m^{-2} s^{-1}; CER_{180}, CER under low PPFD (180 μM m^{-2} s^{-1}, LPFD), μM m^{-2} s^{-1}; T_{1700}, Transpiration rate under HPPFD, mM m^{-2} s^{-1}; T_{180}, transpiration rate under LPPFD, mM m^{-2} s^{-1}; Gs 1700, Stomatal conductance under HPPFD, μM m^{-2} s^{-1}; Gs 180, Stomatal conductance under LPPFD, μM m^{-2} s^{-1}; N, total leaf nitrogen content, %; SLP, soluble protein content, g m^{-2}; FIP, Fraction 1 protein content, mg m^{-2}; PEPC, Phosphoenolpyruvate carboxyase activety, μM m^{-2} s^{-1}; MEMG, Mg type NADP malic enzyme activity, μM m^{-2} s^{-1}; CHL, Chlorophyll content, mg m^{-2}; SLA, specific leaf area, cm^2 d.w.g.$^{-1}$. US56-2, YT/ROC, and YT/Kume shows US56-15-2 of *S. spontanium*, and cv. Yomitanzan (ROC) and cv. Yomitanzan (Kume I.) of *S. sinense*. The level of the statistical significance and student's least significant difference at 5% are indicated by LSD and s-LSD, respectively.

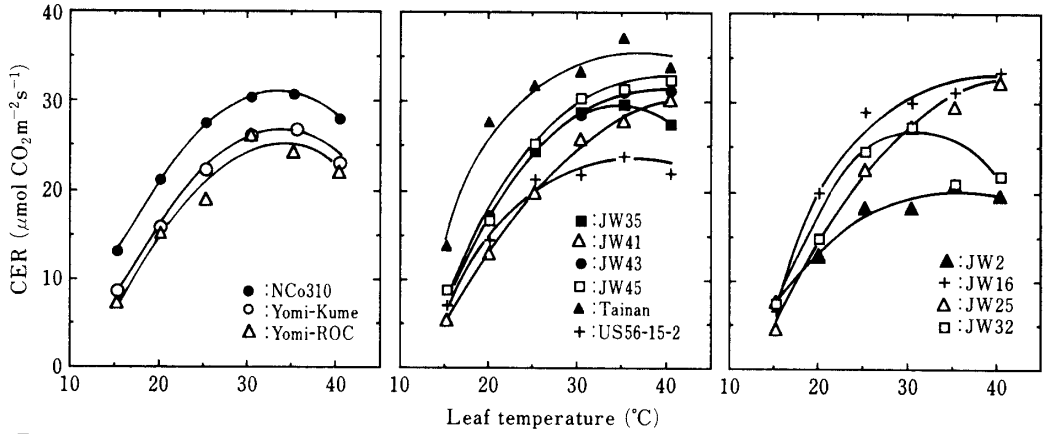

Fig. 1. CO_2 exchange rate (CER) with temperature changes in *Saccharum*.

Variance analysis showed significant differences at the 1% level among lines in each temperature level, and for interaction of line and temperature levels. Student's least significant difference at 5% level was 0.35 and 2.28 μM $m^{-2}s^{-1}$ among temperature levels and lines in each temperature level, respectively.

high and low light intensities, and also showed a significant correlation with malic enzyme activities. However, no significant correlations between leaf conductance and the other traits related to the leaf gas exchange were observed. SLA, which was used to represent the leaf thickness, exhibited a significant negative correlation to total nitrogen content, but had no correlation with the other traits. NADP malic enzyme significantly correlated to many traits related to leaf gas exchange. Chlorophyll content showed a significant positive correlation with CER at high light intensity.

Discussion

CER of *Saccharum* under high light intensity differed significantly among 10 lines of *S. spontaneum* L., *S. sinense* Roxb. cv. Yomitanzan, and *Saccharum* hybrid cv. NCo310. Especially, CER of Tainan, *S. spontaneum* at high light intensity was significantly higher than that of NCo310. The values of CER were higher for

Table 3. Relationship of gas exchange rates to related traits in *Saccharum*.

	CER_{180}	Gs_{1700}	Gs_{180}	N	SLP	F1P	PEPC	MEMG	MEMN	CHL	SLA
CER_{1700}	0.452**	0.496**	—	0.188	0.326*	0.311*	0.128	0.547**	0.838**	0.466**	−0.158
CER_{180}			0.296*	0.030	0.137	0.152	0.075	0.161	0.314**	0.017	0.069
Gs_{1700}			0.576**	0.271*	0.191	0.382**	0.313**	0.478**	0.375**	0.010	0.142
Gs_{180}				0.201	0.124	0.241*	0.199	0.466**	0.352**	−0.093	0.151
N					0.310*	0.320**	0.348**	0.106*	0.048	−0.231	−0.544**
SLP						0.413**	0.297*	0.352**	0.343**	−0.104	−0.073
F1P							0.068	0.399**	0.426**	0.010	0.144
PEPC								0.262*	0.096	0.047	−0.020
MEMG									0.889**	0.356**	−0.153
MEMN										0.338**	−0.196
CHL											−0.275*

Statistical significance at 5% and 1% level are indicated by * and **, respectively. The number of samples ranged from 62 to 75. See Table 2 for abbreviations.

JW2, JW32 and JW41 than for *S. sinense* Roxb. cv. Yomitanzan. Irvine[2] showed that CERs of *Saccharum* hybrids were higher than that of the parent with a low CER, and indicated that CER affected the cane yield. This suggests that *S. spontaneum*, such as Tainan which had a higher CER, is a useful germplasm resource for increasing sugarcane yield.

In the cluster analysis and the principal component analysis[18] carried out in this work, no clear classification among lines, species and collection places was found. These results differ from those observed in *S. spontaneum* collected in the South-West Islands, Japan[13]. It is considered that the sample number in this study is too small to representative of the region, although the materials were selected from wider regions in North-Pacific zones (Table 1).

Tainan, which exhibited the highest CER at high light intensity, also showed the highest rate of CER in the temperature response, in the range of 20 to 35 °C. Temperature in the South-West Islands, including Okinawa, changes seasonally, i.e. in the summer it is similar to that of the Tropics, while in the winter, temperature decreases to the critical level for growth of C_4 plants 14 °C[19]. Since the sugarcane cultivation period varies from 10 to 18 months, the adaptation of CER to low as well as high temperature is important in the South-West Islands. Thus, the temperature response observed in Tainan is considered to be a useful characteristic for the economic sugarcane cultivated in the South-West Islands. In addition, it appeared that *S. spontaneum*, such as JW2, JW32 and US56-15-2, which did not show increased CERs at temperatures higher than 30 °C, would not be suitable as a germplasm resource for improving the sugarcane cultivars in the Tropics.

In this study, no relationship was found between optimum temperature of CER and the temperature conditions of the place of origin. It was not clear why the temperature response of CER in this study differed among *Saccharum*. The rate of leaf gas exchange may be regulated by stomatal aperture and internal leaf factors. Leaf conductance at 30, 35 and 40 °C, however, showed a significant difference ($P<0.05$) between the lines and species. At those temperatures, JW2 and US56-15-2, possessing low CER, showed lower leaf stomatal conductances from 90 to 140 μM $m^{-2}s^{-1}$. However, leaf conductance of JW32 was intermediate, 160 to 200 μM $m^{-2}s^{-1}$. In addition, leaf stomatal conductances of Tainan and NCo310 were also higher, 180 to 230 μM $m^{-2}s^{-1}$ and 150 to 190 μM $m^{-2}s^{-1}$, respectively. Therefore, differences of the temperature response of CER at temperatures higher than 25 °C may be attributed partially to the temperature response of leaf stomatal conductance.

CER is affected by the mesophyll resistance, which is also influenced by the internal CO_2 concentration, which is in turn dependent upon stomatal conductance. McWilliams and Feral[4] have shown that the cold tolerance of the C_4 plant was related to the temperature response properties of PEPCase. Sugiyama and Boku[15] and Sugiyama and Yamazaki[17] also showed that the temperature adaptation of *Zea mays* cultivars developed at different locations was related to the temperature response characteristics of pyruvate orthophosphate dikinase (PPDK) regulating C_4 photosynthesis. Furthermore, Nose et al.[10]

observed that the temperature responses of PEPCase and NADP malic enzyme differed between *S. officinarum* and *Saccharum* hybrids and Rf values of both enzymes, which were separated by electrophoresis and detected by the active staining method, also differed significantly[10]. These results appear to indicate that the differences of the temperature response of CER in *Saccharum* may be caused by differences in the temperature response of the photosynthetic enzymes.

In the study on gas exchange traits of *Saccharum*, what interests us is that which trait plays an important role in the C_4 photosynthesis. We examined 12 traits including CER and other physiological and biochemical characteristics. CER at high light intensity is strongly controlled both by leaf conductance and by internal leaf factors. The same results were also obtained in a study on the effects of nitrogen and potassium nutrients on sugarcane CER[9] and in a study on CER variations in *S. spontaneum*[13]. A close relationship between CER and stomatal conductance was also observed in Irvine's study on CER in *S. officinarum* and *Saccharum* hybrids[2]. In any case, it is considered that stomatal conductance plays an important role in CER of *Saccharum*.

About the internal trait of C_4 photosynthesis, Usuda[20] and Usuda et al.[21] have identified the important role of PPDK, and Sugiyama et al.[16] observed that PEPCase played an important role in photosynthesis. We could not find any significant correlation between PEPCase and CER in *Saccharum*. In this study, however, malic enzyme activity was highly correlated with CER. In the previous study[13] using *S. spontaneum* collected in the South-West Islands, Japan the relationship between NADP malic enzyme and CER did not show any significance in simple correlation. But the relationship was found to be significant in the multiple regression analysis. Thus it seems likely that the significant simple correlation between malic enzyme and CER observed in this study may result from geographic and climatic differences of the collection area. Therefore, it was also observed in the C_4 photosynthesis of sugarcane that malic enzyme showed a close relationship to CER at high light intensity. Although investigation of the role of PPDK in *Saccharum* remains a task for future studies, based on the results of this experiment, the following points are considered to be properties of *Saccharum*: Malic enzyme and fraction 1 protein located in vascular bundle sheath cells (BSC) of sugarcane, and grana in BSC of sugarcane that is not fully developed. Results obtained in this study, such as a close correlation of CER with malic enzyme, fraction 1 protein, or chlorophyll content, may indicate that CER of *Saccharum* was controlled by the photosynthetic efficiency in BSC.

Irvine[2] noted a significant correlation between CER and leaf thickness, directly measured, but not between CER and specific leaf area (SLA). In this study, SLA was obtained from the leaf blade except the mid-rib. CER also was not correlated to SLA. In addition, SLA showed a close and negative relationship to total nitrogen content, but did not correlate with the other traits. The relationship between total nitrogen content and the other internal leaf traits was not strong. It appeared that the relationships between CER and SLA, and between CER and total nitrogen content in *Saccharum*, differed from the relationships found in rice[3]. In the experiment of the nitrogen nutrition effects on CER of *Saccharum* hybrids, there were optimum curves between fraction 1 protein and the total nitrogen content in leaf[10]. These results suggested that the physiological traits regulating CER in *Saccharum* changed independently of total nitrogen content in leaf. It will be interesting to discover which physiological traits regulate CER.

As described above, it was confirmed again in this study that CER and related traits showed significant differences in *Saccharum*. Especially, Tainan, *S. spontaneum* collected in Taiwan, possessed a higher CER, similar to that of JW66, *S. spontaneum* collected in Miyako Island[13]. In addition, it was found that Tainan showed a higher CER in temperature response for a wider range from 20 to 35°C. Therefore, it is considered that these lines of *S. spontaneum* may be useful gene resources for sugarcane breeding in the future.

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