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Changes in leaf photosynthesis and ribulose-1, 5-bisphosphate carboxylase from anthesis through maturation in *Oryza sativa* L.[1,2]

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Abstract. The changes of photosynthetic rate, ribulose-1, 5-bisphosphate carboxylase (RuBPCase) activity, RuBPCase protein content, chlorophyll and soluble protein contents of the upper-most three leaves and the distribution of nitrogen in a tiller were studied from the day of flowering until panicle maturity to understand the relation between leaf photosynthesis and grain growth in the rice plant. The photosynthetic rates of oxygen evolution of the flag leaf reached its maximum at 16 days after flowering and maintained a fairly activity thereafter, while the rate of the third leaf counted down from the top reached its maximum at 5 days after flowering and then senesced. Photosynthetic rate was significantly correlated with RuBPCase activity. RuBPCase activity was positively correlated with RuBPCase protein (r=0.936, n=21) and buffer-soluble leaf protein content (r=0.948, n=21). However, there was a fall in photosynthetic activities during the active grain-filling stage, indicating the change of plant metabolism. The ratio of RuBPCase activity/RuBPCase protein content remained fairly constant throughout the the entire experimental period, suggesting that the photosynthetic activities of leaves were regulated via its RuBPCase protein content. RuBPCase activity was also well correlated with leaf chlorophyll content, but with a lower level of correlation coefficient of r=0.846. The results imply that if the leaf nitrogen content could be maintained or increased during the period of grain-filling, photosynsthetic activities of leaves may be enhanced and hence be favourable for grain growth in the rice plant.

Key words: Chlorophyll; Grain filling; Leaf photosynthesis; Oryza sativa L.; Ribulose bisphosphate carboxylase; Soluble protein.

Introduction

Monocarpic senescence is common in annual cereal crops during the grain-filling period (Dalling, 1985). It is well recognized that during a major part of the grain-filling stage the grain was growing at a faster rate than the entire shoot (including the grain) (Moss, 1976; Shieh, 1979). Therefore, the carbohydrate and nitrogen requirements of the developing grains are largely satisfied by mobilization of carbohydrates (Shieh, 1979; Yoshida, 1972) and protein (Peoples *et al.*, 1980; Simpson *et al.*, 1983; Watars *et al.*, 1980; Wittenbach, 1979; Wittenbach *et al.*, 1980). In the field-grown paddy rice plant, Shieh (1978) observed that, in addition to carbohydrate, the accumulation of nitrogen in developing grains was insufficiently

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accounted by the supply of the currently absorbed and reduced nitrogen from the soil during grain filling and needed to be provided from the redistribution of nitrogen assimilated during the vegetative stage of development. Leaf is the major source of nitrogen in the rice plant. The chloroplast enzyme ribulose-1,5-bisphosphate carboxylase (RuBPCase) is both the key enzyme in photosynthesis (Makino *et al.*, 1983, 1984, 1985) and the major leaf protein (Mae *et al.*, 1982, 1983) in the rice leaf. Prior to senescence, RuBPCase functions to provide photosynthate to the growing regions. During senescence, the degradation of RuBPCase provides additional nitrogen and carbon for mobilization to seed. Because of this dual function there appears to be a conflict within the plant as the protein necessary to maintain photosynthesis is also degraded in the leaf to supply nitrogen for the developing grains. Thus, the regulation of RuBPCase synthesis, degradation and activitity becomes of obvious importance in the provision of reduced carbon and nitrogen to the grain.

In this paper, the experiment was designed to investigate the changes of photosynthetic activities of the upper-most three leaves of the rice plant (*Oryza sativa* L.) during the period from anthesis until grain maturity.

Materials and Methods

Plant Material

The field-grown *japonica* rice (*Oryza sativa* L. cv. Tainung 62) in the first crop season was used as plant material. The experiment was conducted at the experimental farm of the Academia Sinica at Nankang, Taipei. The conventional field management practices were adopted. Single tillers of even growth stems of the mother tiller and the primary tillers were tagged at the day of flowering. Samples of single tillers were collected beginning at the day of flowering and ending at harvest during the first crop season of 1985.

Growth Analysis

Starting from the day of flowering 10 tillers were cut at the stem joint and collected at each sampling time. Each tiller was separated into flag leaf blade (designated as the first leaf), second leaf (leaf immediately below the flag leaf), third leaf, other leaves, culm (stem+leaf sheath) and panicle. After measurements of leaf fresh weight and leaf area, the plant materials were immediately dried in an oven at 100°C for one hour and then two days at 80°C. After drying the dry weight of each plant part was weighted. The dried materials were used for the analysis of total reduced nitrogen. From the data of leaf area and dry weight of organs, growth analysis was carried out using the method of Shieh (1977).

Measurements of Photosynthetic Rate

Different tillers were used to measure the photosynthetic rate of the upper-most three leaves (i.e., the first, second and third leaves) of a tiller at every sampling time. Photosynthetic measurements were made using a Hansatech leaf disc oxygen electrode unit to trace the O_2 exchange of leaves as described by Delieu and Walker (1981). Two leaf segments were cut from a leaf at the middle position by a leaf cutter (3.57 cm in diameter). The leaf segments were floated on 25 mM HEPES buffer (pH 7.0) containing 0.5 mM $CaCl_2$. Prior to the measurement of O_2 exchange, the leaf segments were pre-illuminated with GE 300PAR56/2MFL cool-beam incandescent lamps at 787 μmol m^{-2} s^{-1} PAR for at least 30 min. O_2 exchange was determined for the two leaf segments at 28°C. The oxygen concentration of 21% (v/v) of the atmosphere air was taken for calibration. The leaf segment was illuminated through the roof of the leaf disc chamber by means of a 250W-MS quarz halogen lamp (EYE, Japan) with JAN-O housing, the light beam passing through a spherical flask containing 1% $CuSO_4$ solution. The light intensity

at the leaf surface was measured using a LI-190S Quantum sensor and LI-188B Quantummeter. The photosynthetic photon flux density was 1350 μmol m^{-2} s^{-1} PAR (400-700 nm). After O_2 exchange measurements, one leaf segment was used to assay for chlorophyll content, and the other for total reduced nitrogen content. At least three tillers were made to measure O_2 exchange rates.

RuBPCase Activity Assay

Assays of the RuBPCase activity were made on the leaves of different batches of tillers. Leaves of three upper-most node positions were assayed. RuBPCase activity was determined by following $^{14}CO_2$ incorporation into acid-stable products as described previously (Shieh and Liao, 1985). A sample of two leaves of the same node position was cut into segments and ground on ice with a motor and a pestle and small amount of sea sand in a buffer consisting of 25 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 1 mM Na_2-EDTA and 2% (w/v) PVP. The extract was filtered through 80 μ nylon net and centrifuged at 10000 ×g for 10 min, and the supernatants were directly used to assay for RuBPCase activity. The reaction mixture consisted of 50 mM tricine buffer (pH 7.8), 20 mM $MgCl_2$, 5 mM DTT, 20 mM $NaH^{14}CO_3$, 1 mM RuBP and the enzyme extract in a total volume of 200 μl at 30°C. The same extracts were also used to determine for chlorophyll, total free amino-nitrogen, buffer-soluble protein, and levels of RuBPCase protein. Two leaf samples were assayed for each node-position leaf.

Quatification of RuBPCase Protein

Preparation of Tobacco Leaf RuBPCase: Crystalline RuBPCase was prepared from the fully expanded leaves of two-month old tobacco (*Nicotiana tabacum* L.) following the techniques described by Kung *et al.* (1980). Essentially, 400 g of tobacco leaves was homogenized in 200 ml of 2.0 M NaCl plus 10 mM 2-mercaptoethanol in a Warning blendor. After heated, filtration and centrifugation at 48000×g for 10 min, the supernatant was passed through a Sephadex G-50 column (about 2600 ml bed volume) equilibrated with Tris-EDTA buffer. The collected fraction (about 500 ml) was settled at 4°C to crystallize the enzyme protein. The enzyme was recrystallized 3 times. The cyrstalline RuBPCase was homogeneous as judged by polyacrylamide gel electophoresis (PAGE) and SDS-PAGE (Laemmli, 1970). The specific activity of RuBPCase as assayed by the modified spectrophotometric assay method (Lilley and Walker, 1974) was 0.73 μmoles CO_2 min^{-1} (mg protein)$^{-1}$.

Immunochemical Quantitation of Rice RuBPCase Protein: Antisera against tobacco RuBPCase were raised in three albino rabbits (purchased from the Animal Center of the Medical College, National Taiwan University) using 3-time recrystallized tobacco RuBPCase. The rabbits were injected subcutaneously 3 times with 5 mg antigen resuspended in emulsified Freund's complete adjuvant for the first injection and incomplete Freund adjuvant for the subsequent injections. One week later, antisera of sufficient titer were collected and stored at −27°C in cryovials. Rabbit antibody prepared against tobacco RuBPCase showed a single sharp precitin line when tested angainst antigens prepared from crystalline tobacco RuBPCase and the crude extracts of tobacco and rice leaves by the Ouchterlony double diffusion technique (Fig. 1). The homogeneity of the antigens was further demonstrated by immunoelectrophoresis which revealed a single arc.

The levels of RuBPCase protein in leaves at the three node positions of rice tillers were determined by single radial immunodiffusion (Vaerman, 1981). On a GelBond film (Pharmacia Fine Chemicals, Sweden) (82×63×1.6 mm) containing 1% agarose in buffer consisting of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.9% NaCl and 0.1% sodium azide, and the antiserum with final dilution of 1:60, eight microliters of cyrstalline tobacco RuBPCase (70 to

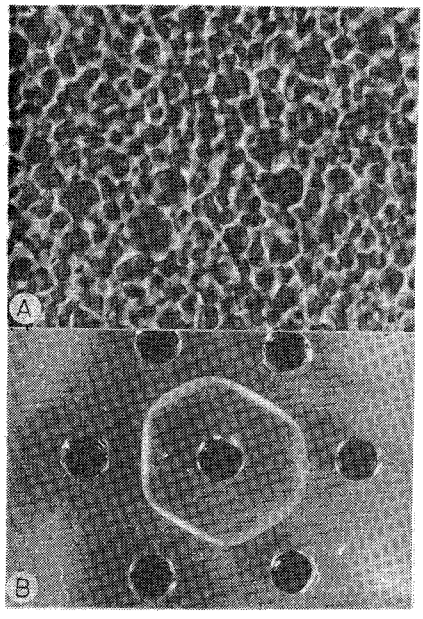

Fig. 1. (A) Crystalline tobacco leaf RuBPCase protein, and (B) Ouchterlony double immunodiffusion of extracts of rice leaves aganist the rabbit antiserum.

420 μg/ml) or rice leaf extracts were placed in wells with diameters of 3 mm, then the gel was incubated for 72 h at room temperature for diffusion in a humid chamber. The gel was then washed with saline phosphate solution consisting of 0.04 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4 and 0.02% sodium azide, dried, and then stained with Coomassie Brilliant Blue R-250. The diameter of each radial precipitin zone was measured under dark field illumination using a vernier caliper (Mitutoyo, Japan) precise to 0.01 mm, aided by a 12 cm diameter 3× magnifier.

Nitrogen, Chlorophyll, Protein and Amino-nitrogen Anaylsis

Total reduced nitrogen of the plant parts was determined in powdered samples as described (Shieh, 1978). Buffer-soluble protein was determined by Coomassie Brilliant Blue G-250 dye-binding method of Spector (1978) with bovine serum albumin as standard. Free amino-nitrogen was determined by the method of Yemm and Cocking (1955) using glycine as standard. Chlorophyll was determined after Wintermans and De Mots (1965).

Results and Discussion

Tiller and Panicle Growth

The patterns of tiller growth (including the grain) and panicle growth for *Oryza sativa* L. cv. Tainung 62 are shown in Fig. 1A. After an exponential phase of approximately 16 days, the rate was linear from day 23 after flowering (DAF) to maturity. The average panicle weight at harvest was 2.65 g. The pattern of grain growth recorded in this experiment is similar to those

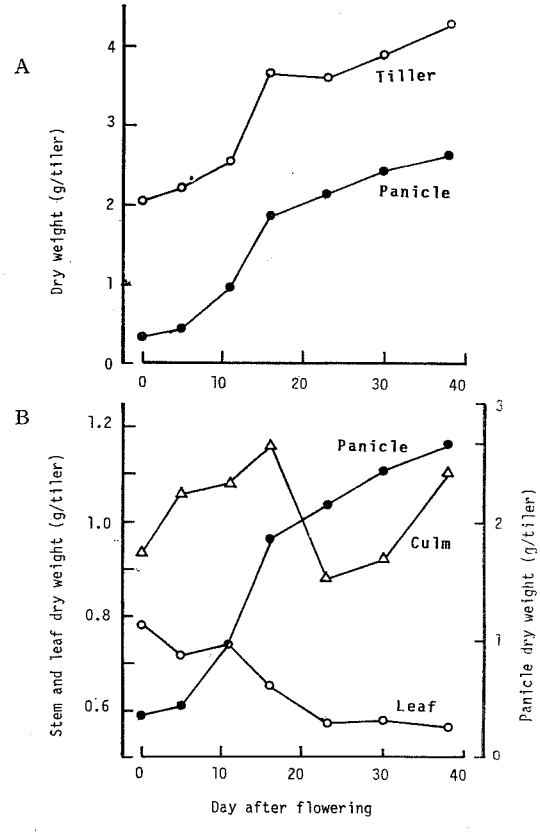

Fig. 2. (A) Changes in the tiller (○) and panicle (●) dry weight during grain development. (B) Changes in leaf (○), culm (△), and panicle (●) dry weight during grain development from anthesis to maturity.

patterns observed previously (Shieh, 1979). Fig. 1B shows the changes of the culm and leaf dry weights during grain development. The total leaf dry weight of a tiller decreased after 10 DAF. The culm dry weight increased at the early stage of grain growth until 16 DAF. It is interesting to see that there was a fast decline of the culm dry weight during the period from 16 to 23 DAF, and then the dry weight raised again afterwards. The declines in culm and leaf dry weights may indicate the remobilization of material from the senescing organs and the redistribution of nitrogen occurs prior to carbohydrate.

Changes in Leaf Dry Weight, Leaf Area, Chlorophyll, Amino-N, and Protein at Individual Node Position

The dry weight of the flag leaf (leaf 1), after an increase from anthesis to 11 DAF, changed little (Fig. 3A). The increase in dry weight of

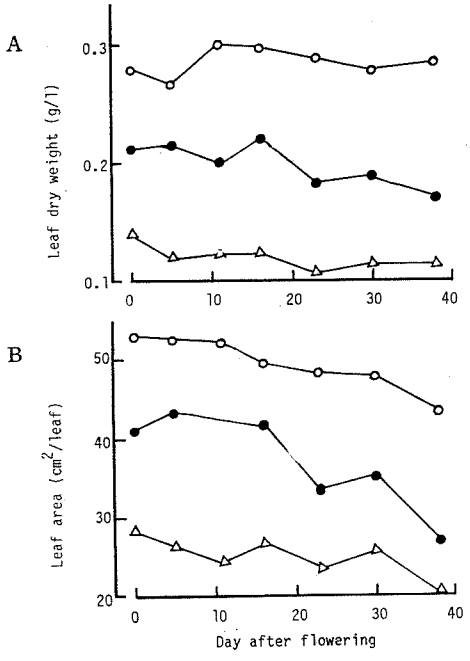

Fig. 3. Changes in leaf dry weight (A) and leaf area (B) of leaves at different node postions during grain growth from anthesis to maturity. ○, the flag leaf; ●, the second leaf; △, the third leaf.

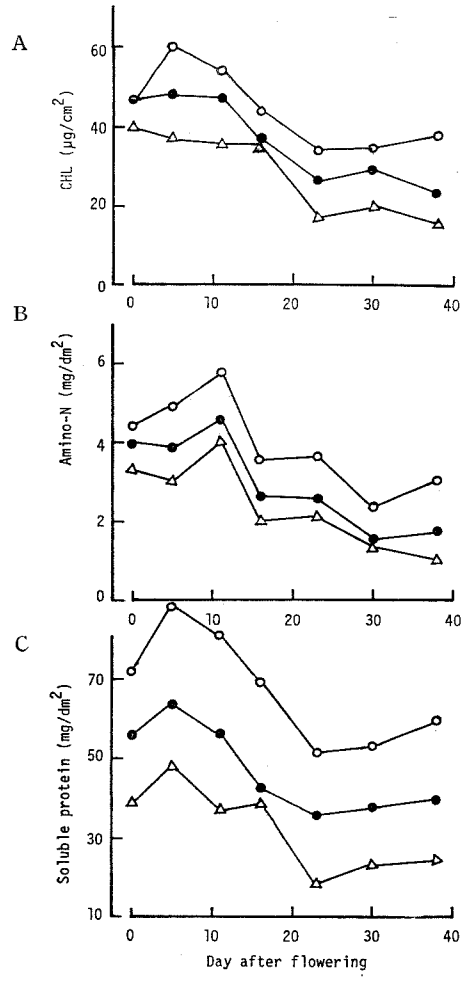

Fig. 4. Changes in (A) chlorophyll, (B) free amino-N, and (C) buffer-soluble protein contents on an area basis during grain development. The symbols are the same as in the legend of Fig. 3.

the flag leaf may be due to increasing in photosynthesis during this period (Fig. 5A). The dry weight of the leaf 2 changed little from 0 to 16 DAF until the start of senescence. The leaf area of the leaf 1 and leaf 2 decreased after 16 DAF (Fig. 3B). The dry weight and leaf area of the third leaf decreased from the day of anthesis, indicating maturation of this leaf was before anthesis.

Figure 4 shows changes in leaf chlorophyll, amino-N, and buffer-soluble protein. The chlorophyll content of the flag leaf declined later than

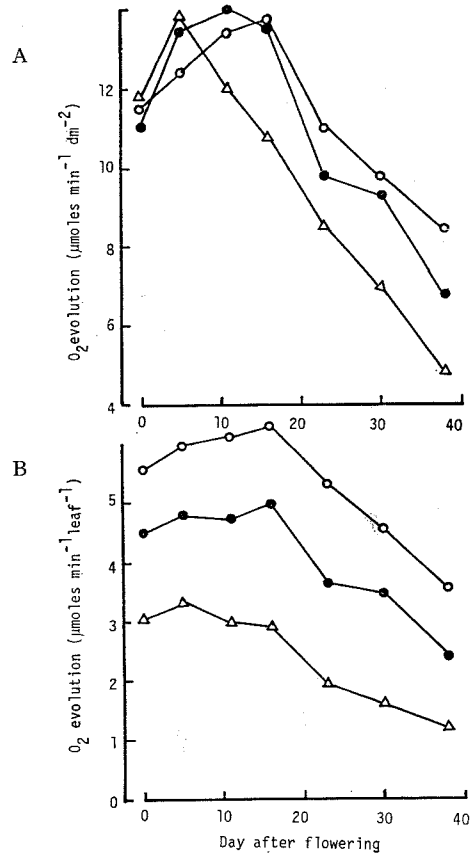

Fig. 5. Changes in photosynthetic rates of oxygen evolution on an area (A) and leaf blade (B) basis. The symbols are the same as in Fig. 3.

those in the second and third leaves (Fig. 4A). The soluble protein content increased just prior to 5 DAF, followed by a rapid decline, and then leveled off after 23 DAF (Fig. 4C). The onset of protein degradation occurred earlier than that of chlorophyll (Makino *et al.*, 1983). The changes of amino-N content occurred 5 days later than the breakdown of protein, followed by a rapid decline in amino-N (Fig. 4B). The degradation of protein coincidented with the beginning of the exponential phase of the grain growth. The decline of amino-N ocurred during the linear period of the exopential phase. Since no large increase in amino-N were assoicated with the lose of protein from the leaves, it may indicate that the breakdown products were being rapidly translocated out of the leaves to the growing regions. Since the major sinks at this time are the developing seeds, which are rapid accumulating nitrogen (Shieh, 1978), it is resonable to assume that this protein-N is being translocated to the grains.

Changes in Photosynthesis and RuBPCase

The photosynthetic rates of oxygen evolution on an area basis are shown in Fig. 5A. The maximal activity of the three node-positioned leaves was apparently similar. However, the time to reach their maximal rates were different.

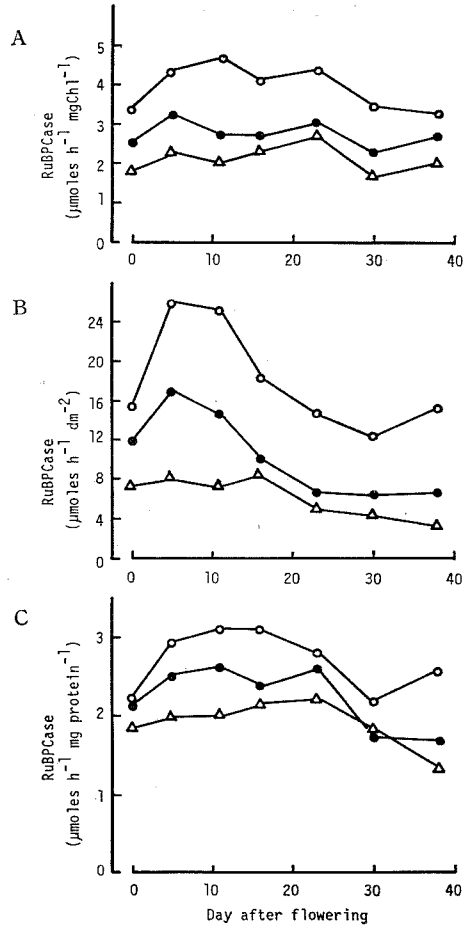

Fig. 6. Changes in RuBPCase activity on chlorophyll (A) and an area (B) basis, and the specific RuBPCase activity (C) during grain development from anthesis to maturity. Symbols are the same as in Fig. 3.

There was a rise in photosynthesis in the flag leaf until 16 DAF, and declined rapidly. The rate of the second leaf reached its maximum at 5 DAF, then leveled off and began to decline rapidly about 16 days after flowering. The photosynthetic rate of the third leaf began to decline about 10 days prior to those in the flag and second leaves, and a paralleled decline was observed in the upper leaves. When the photosynthetic oxygen evolution was presented as on the basis of whole leaf blade, the contribution of leaf photosynthesis at different node-positions to grain growth became apparent (Fig. 5B), while the tendencies of the changes in photosynthetic activity were similar to those shown in Fig. 5A. A good correlation existed between the decline in photosyntetic activity associated with senescence and the decline in RuBPCase activity for the leaves (Fig. 6B). Moreover, the changes in carboxylase activity very closely followed the changes in protein levels on an area basis, while the RuBPCase activities on chlorophyll or soluble protein basis were maintained fairly constant (Fig. 6A and 6C), as observed in the seedling of the rice plant (Shieh and Liao, 1987).

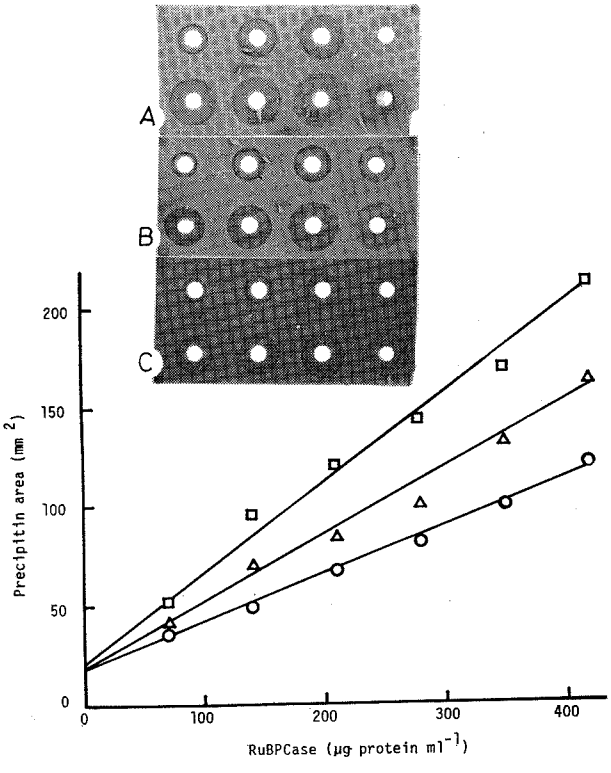

Fig. 7. Measurement of RuBPCase protein content by single radial immunodiffusion. Standard curves showing the relationship between the quantity of standard RuBPCase protein and precipitin area. A, B, C represented the dilution of rabbit antisera at 1:100 (□), 1:60 (△), and 1:40 (1:40 (○), respectively.

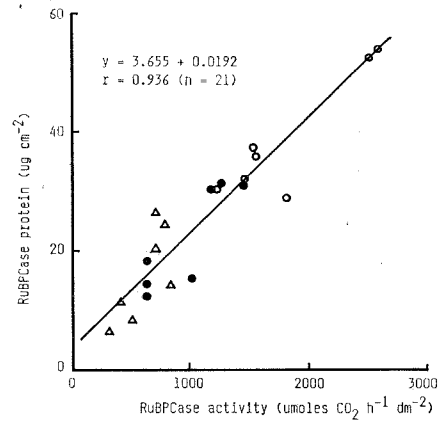

Fig. 8. Correlation of RuBPcase protein aganist RuBPCase activity. The regression line was based on the pooled data of all leaves during grain development from anthesis to harvest. The symbols are the same as in Fig. 3.

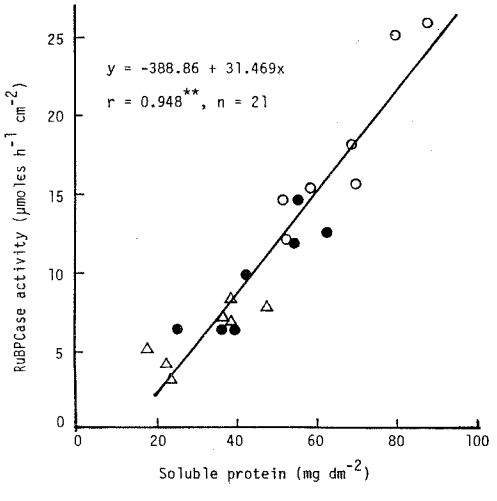

Fig. 9. Correlation between RuBPCase activity and soluble protein. The illustration is the same as in the legend of Fig. 8.

Using rabbit antiserum against tobacco leaf RuBPCase protein, the single radial immunodiffusion was conducted to measure the levels of RuBPCase protein in rice leaf extracts. The antiserum allowed quantification with known amounts of crystalline tobacco leaf RuBPCase used as the standard (Fig. 7). The contents of RuBPCase in rice leaf extracts were well correlated with the RuBPCase activities (Fig. 8), having a correlation coefficient of 0.936 (n=21). Similar obserbation was also found for a single leaf (Makino *et al.*, 1984, 1985).

Correlations between Leaf Photosynthetic Parameters

Table 1 shows correlations of different parameters related to the leaf photosynthesis of the rice plant. The RuBPCase activity was significantly correlated with soluble protein content (r=0.948, n=21) (Fig. 9) and chlorophyll content (r=0.891, n=21) (Fig. 10). The soluble protein

Table 1. *Correlation coefficients and regression equations betweem photosynthetic related parameters*

The correlations were made for the data from same leaf extracts or from different leaf samples of the same node positions of the three leaves.

Correlation[a]		Regression function	r^b
N% from leaves of O_2 evolution data			
Chl (O_2)	vs N%	CHL=3.721+17.859 (N%)	0.935
Chl (enzyme)	vs N%	CHL=−2.053+15.559 (N%)	0.801
Solube protein	vs N%	Sol-P=−13.257+23.968 (N%)	0.755
RuBPC protein	vs N%	RuBPC-P=−15.602+15.956 (N%)	0.737
RuBPC activity	vs N%	RuBPC-A=−722.77+722.17 (N%)	0.686
O_2 evolution	vs N%	Poxy=2.759+3.125 (N%)	0.805
CHL from leaves of O_2 evolution data			
Solube protein	vs CHL	Sol-P=−7.935+1.136 (CHL)	0.683
RuBPC protein	vs CHL	RuBPC-P=−10.930+0.734 (CHL)	0.647
RuBPC activity	vs CHL	RuBPC-A=−529.158+33.569 (CHL)	0.609
O_2 evolution	vs CHL	Poxy=1.927+0.179 CHL)	0.878
CHL from leaves of enzyme data			
Soluble protein	vs CHL	Sol-P=−8.268+1.493 (CHL)	0.913
RuBPC protein	vs CHL	RuBPC-P=−12.268+0.994 (CHL)	0.891
RuBPC activity	vs CHL	RuBPC-A=−606.832+45.877 (CHL)	0.846
O_2 evolution	vs CHL	Poxy=5.811+0.133 (CHL)	0.661
RuBPC protein	vs Sol-P	RuBPC-P=−5.601+0.642 (Sol-P)	0.940
RuBPC activity	vs Sol-P	RuBPC-A=−388.86+31.469 (Sol-P)	0.948
O_2 evolution	vs Sol-P	Poxy=7.156+0.07645 (Sol-P)	0.622
RuBPC-A	vs RuBPC-P	RuBP-A=−24.048+45.548 (RuBPC-P)	0.936
O_2 evolution	vs RuBPC-P	Poxy=8.252+0.103 (RuBPC-P)	0.570
O_2 evolution	vs RuBPC-A	Poxy=8.430+0.002144 (RuBPC-A)	0.580
RuBPC-P	vs RuBPC-A	RuBPC-P=3.655+0.01924 (RuBP-A)	0.936
CHL (O_2)	vs CHL (enzyme)	CHL (O_2)=20.555+0.7704 (CHL)	0.783

[a] N%, total reduced nitrogen content of leaf; CHL, chlorophyll; RuBPC-A, RuBPCase activity; RuBPC-P, RuBPCase protein; Sol-P, soluble protein; Poxy, photosynthetic O_2 evolution.

[b] Correlation coefficient, r=0.549 (n=21) at P=0.01.

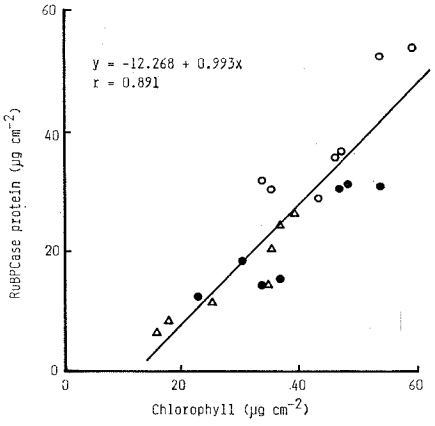

Fig. 10. Correlation between the level of RuBPCase protein and chlorophall content. The illustration is the same as in the legend of Fig. 8.

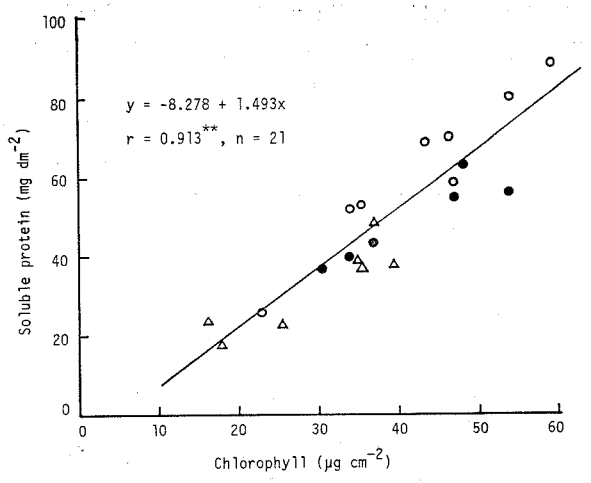

Fig. 11. Correlation beetween buffer-soluble protein and chlorophyll content on an area basis.

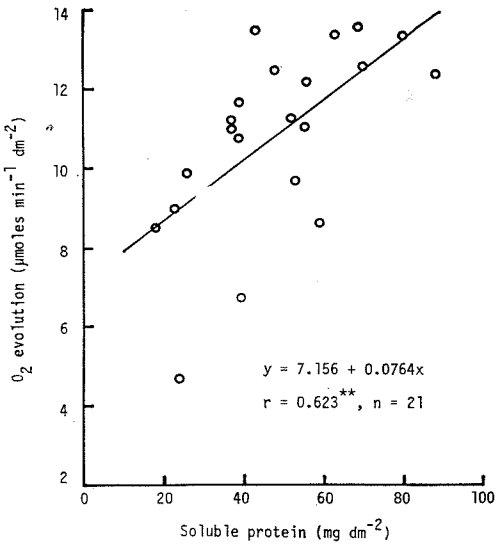

Fig. 12. Correlation between photosynthetic oxygen evolution and buffer-soluble protein content on an area basis.

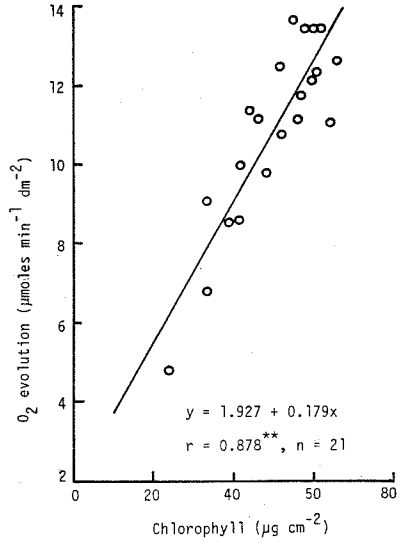

Fig. 13. Correlation between photosynsthetic oxygen evolution and chlorophyll content on an area basis during grain development from anthesis to harvest.

was also well correlated with chlorophyll with $r = 0.913$, $n=21$ (Fig. 11).

The photosynthetic oxygen evolution was positively correlated with soluble protein (Fig. 12, Table 1) and chlorophyll (Fig. 13).

It appeared that the relationships between photosynthetic activites were higher when measured from the same leaf, while the correlation coefficients were lower for the parameters taken from different batches of leaves. Since RuBPCase activity and the photosynthetic oxygen evolution were assayed separately from different leaves at the same node position, the correlation coefficient was rather low ($r=0.58$, $n=21$) (Table 1). For example, the correlation coefficients between photosynthetic oxygen evolution and chlorophyll

content was 0.878, while $r=0.661$ as obtained from different leaves. The experiments showed that good results could be obtained when photosynthesis related parameters were measured from the same leaf source. Nevertheless, these correlations are statistically significant at the 1% level.

Discussion

The results in this experiment demonstrated that the photosynthetic oxygen evolution on an area basis is well related with RuBPCase activity and the fraction I protein content. Since these relationships were calculated from the pooled data of all three leaves of a tiller, it may indicate that the photosynthetic carbon assimilation is in good agreement with the content of leaf RuBPCase protein in rice plant (Makino *et al.*, 1983, 1985)). It is apparent that there was no inhibition of RuBPCase activity in leaves to limit carbon fixation. The changes in the photosynthetic oxygen evolution was well correlated with other photosynthetic parameters, particularly RuBPCase. The decline of photosynthesis during active grain-filling period occurred coincidently with the declines of soluble protein and RuBPCase protein, and the decrease of soluble protein occurred earlier than photosysnthesis. It may indicate that during active grain filling the seed is calling for nitrogen and carbohydrate and needs the remobilization of the material from vegetative regions. Similar results were observed in other cereals (Peoples *et al.*, 1980; Simpson *et al.*, 1983; Waters *et al.*, 1980; Wittenbach, 1979; Wittenbach *et al.*, 1980). The chloroplast enzyme RuBPCase plays bifunctional role in the rice leaf as the key enzyme in photosynthesis (Makino *et al.*, 1983, 1984, 1985) and the major leaf protein. Monocarpic senescence is an inevitable adaptation in cereal crops, and the efficient mobilization of essential and often limiting nutrients from the the vegetative organs to the seed is of inestimatable economic importance. However, it occurs early during the period of the grain developing stage in the rice plant. The results in this experiment imply that if the leaf nitrogen content could be maintained or increased during the period of active grain filling, photosynthetic activities of leaves may be enhanced and hence be favourable for grain growth in the rice plant,

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水稻穀粒充實期間光合作用與雙磷酸核酮糖羧化酶的變化

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自開花日到穗成熟調查粳稻品種臺農 62 號分蘗包含劍葉之最上三葉片的光合作用氧氣釋放速率、雙磷酸核酮糖羧化酶 (RuBPCase) 活性及其蛋白質量，葉綠素，全氮量及可溶性蛋白質量，以瞭解在稻穀充實期間之光合作用與穀粒生長的關係。水稻之劍葉之光合作用速率在開花後 16 日達最高，期後仍保持相當的活性，而自上下數第三葉在開花後 5 日已達最高速率，其後即逐漸老化。在整個成熟期間，葉之光合作用速率與 RuBPCase 活性呈正相關，並且 RuBPCase 活性與 RuBPCase 蛋白質 (r=0.936) 及可溶性蛋白質 (r=0.948) 呈正相關。然而，在穀粒充實期中發現光合作用活性有下降的現象，指示分蘗稈代謝作用的改變，而 RuBPCase 活性與 RuBPCase 蛋白質的比例在整個時期保持相當穩定，提示水稻葉之光合作用主要由葉之 RuBPCase 蛋白質所調節。RuBPCase 活性亦與葉綠素含量呈正相關，但相關較低，r=0.846。此結果暗示在穀粒充實期間若能保持或增進葉的含氮量，可望提高水稻的光合作用，進而有利於稻穀的充實。