Photosynthesis, Dark Respiration and Protein Synthesis of Rice Leaves at Low Temperature —Analysis of Ribulose-1,5-Bisphosphate Carboxylase*—

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Abstract : The effects of low temperature on photosynthesis, dark respiration and protein synthesis in developing fourth-leaf blades of rice (*Oryza sativa* L.) seedlings were investigated. Photosynthesis and respiration were measured with an oxygen electrode system, and protein synthesis was analyzed by SDS-polyacrylamide gel electrophoresis, fluorography and immunoprecipitation of ribulose-1,5-bisphosphate carboxylase (RuBPC) after *in vivo* labeling of leaf proteins with [³⁵S]methionine. When the rice seedlings were grown at a low temperature of 15°C for 10 days, the photosynthetic rate was severely inhibited, whereas the dark respiration rate of the stressed plants was even higher than the control plants grown at 25°C. The *de novo* synthesis of total proteins was also inhibited by low temperature, and the distribution of synthesized proteins drastically changed with growth and incubation temperatures, especially in the proteins related to photosynthesis. An immunological analysis of RuBPC revealed that synthesis of the RuBPC proteins was suppressed more severely than the other soluble proteins at low temperature, and that the synthesis of large subunits of RuBPC was more sensitive to the temperature treatments than that of small subunits. These results indicate that the synthesis of RuBPC proteins, especially in the part associated with chloroplasts, is specifically susceptible to low temperature, and that this may be one of the causes of retarded photosynthesis and the poor development of leaves on rice plants grown at low temperatures.

Key words : Dark respiration, *Oryza sativa* L., Photosynthesis, Protein synthesis, Ribulose-1,5-bisphosphate carboxylase, Rice.

水稻葉の光合成、暗呼吸およびタンパク質合成に及ぼす低温の影響 —リブローズ-1,5-二リン酸カルボキシラーゼの解析— : 丸山幸夫・中村保典 (農業生物資源研究所)

要 旨 : 水稻幼植物の伸長中の第4葉身を供試し、光合成、暗呼吸およびタンパク質合成に及ぼす低温の影響を調べた。光合成速度および暗呼吸速度は酸素電極法で測定し、タンパク質合成は [³⁵S]メチオニンのタンパク質画分への取り込みを定量するとともに標識されたタンパク質をSDSポリアクリルアミドゲル電気泳動で分離しフルオログラフィーにより解析を行った。25°Cで生長させた水稻幼植物を15°Cで10日間低温処理すると、葉身の光合成速度は著しく低下するが、暗呼吸速度はむしろ対照植物よりも増加した。タンパク質の合成は低温により阻害されたが、合成されたタンパク質の分布は生育温度と処理温度により著しく異なり、とくに光合成機能に関連するタンパク質の合成が低温により阻害される傾向が認められた。そこで、リブローズ-1,5-二リン酸カルボキシラーゼ (RuBPC) について免疫手法を用いて解析したところ、低温下では他の可溶性タンパク質と比較してRuBPCタンパク質の合成が阻害されやすいこと、RuBPCの小サブユニットよりも大サブユニットの合成の方が温度処理の影響を受けやすいことが判明した。これらの結果は、生長、発達中の葉身においてRuBPCタンパク質の合成のうち葉緑体と関連する過程が特異的に低温感受性が高いことを示すものであり、このことが低温下における水稻幼植物の光合成および生長阻害の一因と考えられた。

キーワード : 暗呼吸、光合成、水稻、タンパク質合成、リブローズ-1,5-二リン酸カルボキシラーゼ。

It has been reported that growth and metabolism are seriously inhibited above chilling temperature in the range between about 12°C and 18°C in rice seedlings⁷⁾. Although chilling injury has been well investigated^{4,14)},

the mechanism of growth inhibition at this temperature range above chilling is not fully understood.

In the previous report¹⁶⁾, we investigated the effects of low temperature on the rate of growth, the contents of chlorophyll and proteins, and the levels of several enzyme activities of developing leaves of rice seedlings. The results indicated that key proteins involved in photosynthesis are specifically susceptible to

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low-temperature stress during leaf development.

In the present study, we have measured photosynthetic and dark respiration rates in leaf blades of rice seedlings, in order to confirm the specific failure of cellular functions found in our previous report¹⁶⁾. On the other hand, we have also investigated influences of low temperature on protein synthesis in developing leaves of rice seedlings, adjusting the focus on synthesis of low-temperature susceptible proteins related to photosynthesis, especially synthesis of ribulose-1,5-bisphosphate carboxylase (RuBPC) subunits, separately.

Materials and Methods

1. Plants and growth conditions

Rice plants (*Oryza sativa* L., cv. IR 8) were grown using the method previously described¹⁶⁾. Five germinated seeds were sown in each 0.9-liter plastic pot, and the pots were placed in a growth cabinet at 25°C, 70% relative humidity, and 400 $\mu\text{mol m}^{-2}\text{s}^{-1}$ quantum flux, with a day length of 14 h.

Low-temperature treatment was imposed when the tip of the fourth leaf just appeared from the ligule of the third leaf. This was done at 10 or 11 days after sowing. The potted plants were transferred to the other growth cabinet where the temperature was maintained at 15°C, and the lighting conditions and the humidity were the same as those at 25°C. Some plants continued to grow at 25°C to serve as a control.

2. Measurement of photosynthetic and respiration rates

The blades of the fourth leaves were sampled at 11 a.m. from the control and the stressed plants, 6 and 10 days after the temperature treatments, respectively. Middle part of the leaf blade (ca. 2 cm in length) was further excised with a razor blade rapidly, and five leaf sections were placed in a chamber of a leaf-disk oxygen electrode system (Model LD-2, Hansatech Ltd. Norfolk, UK). The photosynthetic rate was determined by measuring the rate of increase in O_2 at a CO_2 concentration of approximately 10% under a light intensity of 1,000 $\mu\text{mol m}^{-2}\text{s}^{-1}$. The respiration rate was determined by the rate of decrease in O_2 at a CO_2 concentration of 0.03% in the dark. Temperature in the chamber was controlled at 25° and 15°C, alternative-

ly.

3. Labeling conditions and analysis of radiolabeled proteins

The blades of the fourth leaves were cut off at 11 a.m. from the control and the stressed plants, 2 and 6 days after the temperature treatments, respectively. At these sampling times, the increases of leaf proteins were rapid and in linear phases¹⁶⁾. Six leaf sections (ca. 0.5 cm in length) were further excised from the middle part of the leaf blade with a razor blade, and the fresh weight was quickly determined.

In vivo labeling of leaf proteins was performed by a method using leaf sections¹⁹⁾. Three to six leaf sections were placed in a glass tube (0.8 cm in diameter and 5 cm in length) with one cut end dipped in 10 μL of 50 mM Tris-HCl (pH 7.5) supplemented with 0.2 μM [³⁵S]methionine (200 $\mu\text{Ci mL}^{-1}$). Temperature was controlled at 25 or 15°C, and illumination was provided from a 700-W high pressure mercury lamp (120 $\mu\text{mol m}^{-2}\text{s}^{-1}$). After incubation for 1 h, the leaf sections were quickly frozen in liquid N_2 and stored at -80 °C until further analysis.

The leaf samples were ground on ice with fine quartz sand in 50 μL of prechilled extraction buffer that contained 50 mM Tris-HCl (pH 7.5), 1 mM $\text{Na}_2\text{-EDTA}$, 8 mM MgCl_2 , and 2% (v/v) 2-mercaptoethanol. The homogenate was centrifuged at 17,000 $\times g$ for 10 min at 4°C. The supernatant was taken for analysis of soluble proteins. The precipitate was washed twice with 0.5 mL of the extraction buffer to remove soluble proteins. Insoluble proteins were extracted from the precipitate after suspension in 50 μL of 50 mM Tris-HCl buffer (pH 6.8), 8 M urea and 3.2% (w/v) SDS. The suspensions were kept at 40°C for 60 min, and then centrifuged at 17,000 $\times g$ for 10 min. The supernatant was taken for analysis of insoluble proteins.

Ten micro liters of the supernatant containing soluble or insoluble protein fractions were monitored for radioactivity in a liquid scintillation counter (Model LS 3801, Beckman, CA, U.S.A.). Incorporation of [³⁵S]methionine into proteins was determined by precipitating the supernatant with 10% trichloroacetic acid (TCA).

The rest of the supernatant of the soluble or insoluble protein fractions was used for analy-

Table 1. Effects of low-temperature treatment on photosynthesis and dark respiration of fourth-leaf blade of rice seedling (cv. IR 8).

Temperature treatment (°C)	Photosynthetic rate (nmol O ₂ gFW ⁻¹ s ⁻¹)	Respiration rate (nmol O ₂ gFW ⁻¹ s ⁻¹)
25†-25‡	352±21 (100)	10.3±4.1 (100)
25-15	150±14 (43)	5.2±1.1 (50)
15-25	79±31 (22)	20.0±2.3 (194)
15-15	31±13 (9)	13.6±4.0 (132)

†, Growth temperature; ‡, incubation temperature. Values are expressed as mean±S.D. of three to seven individual experiments. The numbers in parenthesis are the relative values to those of control (25°C-25°C).

sis of polypeptides by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)¹². Identical amounts of TCA-precipitated radioactivity were loaded in each lane. Fluorograms were prepared according to the method of Bonner and Laskey².

4. Immunological analysis of RuBPC

Rabbit antiserum raised against the purified horoenzyme of RuBPC in rice leaves²² was used for immunoprecipitation analysis. *In vivo* labeled leaf samples with [³⁵S]methionine, as the same procedure described above, were ground on ice with fine quartz sand in 50 µL of prechilled enzyme extraction buffer that contained 100 mM tricine-NaOH (pH 8.0), 8 mM MgCl₂, 2 mM EDTA, 50 mM 2-mercaptoethanol, and 12.5% (w/v) glycerol. The homogenate was centrifuged at 17,000 × g for 15 min at 4°C. The supernatant was taken, and then added with 20 µL of rabbit antiserum. The mixture was incubated at 30°C for 30 min and centrifuged at 17,000 × g for 10 min. The immunoprecipitate was washed with enzyme extraction buffer twice, and RuBPC was solubilized from the immunoprecipitate after suspension in 10 µL of extraction buffer which contained 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol. The suspensions were heated at 100°C for 3 min, and then loaded onto SDS-polyacrylamide gels. After staining the gels with Coomassie brilliant blue, the bands corresponding to large (LSU) and small (SSU) subunit of RuBPC were cut out. The gel pieces for LSU and SSU were further excised into sections and incubated with 4% of Protosol (NEN, Boston, MA, U.S.A.) in Econofloor (NEN) at 37°C for 3 days. The radioactivity was determined by the liquid scintillation counter described above.

The radioactivity of immunoprecipitated

RuBPC subunits in the SDS-polyacrylamide gels was also directly determined by an automatic radioactive scanner (TLC Multi Tracemaster 20, Berthold, Wildbad, Germany). The stained gels were dried and the radioactivity was scanned with radioactive scanner for 10 min. The intensity of bands of proteins on the same gels was also measured with a gel densitometer (Ultrascan XL Enhanced Laser Densitometer, Pharmacia-LKB, Uppsala, Sweden).

Results and Discussion

1. Effects of low temperature on photosynthetic and dark respiration rates

The rates of photosynthesis and dark respiration of fourth-leaf blades in control (grown at 25°C) and stressed (treated at 15°C for 10 days) plants were measured at 25°C and 15°C, using a leaf-disk oxygen electrode system. When the control plants were incubated at a low temperature of 15°C, the photosynthetic rate was reduced to 43% of the same plants incubated at 25°C (Table 1). The photosynthetic rate of the stressed plants incubated at 15°C was only 9% of the control plants measured at 25°C, whereas the rate increased up to 22% when incubated at 25°C.

Low temperature reduces photosynthetic rate in leaves of rice seedling grown under normal temperature^{6,21}, and low-temperature pretreatment also inhibits photosynthetic rate in developed leaves of rice when measured at a normal temperature^{10,20}. On the other hand, Makino et al.¹⁵ reported that the CO₂-saturated photosynthetic rate of rice leaves was negatively affected by growth temperature. In the present study, photosynthetic rate was severely inhibited in developing leaves of

Table 2. Effect of low-temperature treatment on [^{35}S]methionine incorporation into protein fractions of fourth-leaf blade in rice seedling (cv. IR 8).

Temperature treatment (°C)	Incorporated radioactivity ($\times 10^4$ dpm mgFW $^{-1}$ h $^{-1}$)		
	Soluble	Insoluble	Total
25†-25‡	5.55 $\pm$ 0.53 (100)	10.18 $\pm$ 2.81 (100)	15.74 $\pm$ 2.87 (100)
25-15	1.28 $\pm$ 0.25 (23)	3.07 $\pm$ 1.31 (30)	4.36 $\pm$ 1.31 (28)
15-25	2.79 $\pm$ 0.22 (50)	3.71 $\pm$ 0.26 (36)	6.50 $\pm$ 0.19 (41)
15-15	0.97 $\pm$ 0.26 (17)	1.62 $\pm$ 0.53 (16)	2.59 $\pm$ 0.27 (16)

†, Growth temperature ; ‡, incubation temperature. Values are expressed as mean $\pm$ S.D. of three individual experiments. The numbers in parenthesis are the relative values to those of control (25°C-25°C).

the stressed plants even if measured at normal temperature, indicating that low temperature strongly affects the developmental process of photosynthetic apparatus.

When the control plants were incubated at 15°C, the respiration rate in the dark decreased to 50% of the same plants measured at 25°C (Table 1). However, the stressed plants exhibited an approximately 2-fold higher rate of dark respiration than the controls when incubated at 25°C. It is also noted that the rate of respiration measured at 15°C in the stressed plants was still higher than that measured at 25°C in the control plants. Therefore, the dark respiration rate was maintained at the higher level as compared with photosynthetic rate in the stressed plants. We assume that under low-temperature conditions, the dark respiration plays an important role in the energy metabolism. The higher rate of dark respiration and the lower rate of photosynthesis in the stressed plants as compared with those of the control plants are in consistent with the enzymic activities of intracellular components in our previous report¹⁶⁾.

2. Effect of low temperature on protein synthesis

Protein synthesis in developing fourth-leaf blades of rice seedlings was measured by incubating leaf segments with [^{35}S]methionine in the light. When the control plants were incubated at 15°C, incorporation rate into total proteins was markedly reduced to 28% of the same plants incubated at 25°C (Table 2). Incorporation rate into total proteins of the stressed plants incubated at 15°C was 16% of the control plants measured at 25°C, whereas the rate increased to 41% when incubated at 25°C. Decrease of incorporation rate into sol-

Fig. 1. Fluorograph of [^{35}S]methionine labeled leaf extracts separated by SDS-polyacrylamide gel electrophoresis.

Lanes 1-4 and 5-8 correspond to the soluble and the insoluble protein fractions, respectively. Lanes 1 and 5, 25°C (growth temperature) -25°C (incubation temperature) ; lanes 2 and 6, 25°C-15°C ; lanes 3 and 7, 15°C-25°C ; lanes 4 and 8, 15°C-15°C.

uble proteins by low incubation temperature was larger, and that by low growth temperature was smaller, as compared with insoluble proteins.

Fluorograph of [^{35}S]methionine-labeled proteins in leaf blade extracts separated by SDS-PAGE was shown in Figure 1. Obvious temperature-dependent changes were observed in the distribution of the radioactivity incorporated into the soluble and insoluble protein fractions. When the control plants were subjected to a low temperature of 15°C, LSU of RuBPC with the molecular size of 55

Table 3. Effect of low-temperature treatment on [^{35}S]methionine incorporation into large subunit (LSU) and small subunit (SSU) fractions of immunoprecipitate for RuBPC of fourth-leaf blade in rice seedling (cv. IR 8).

Temperature treatment (°C)	Incorporated radioactivity ($\times 10^4$ dpm mgFW $^{-1}$ h $^{-1}$)			
	LSU	SSU	Total	LSU/SSU
25†-25‡	2.63 $\pm$ 0.50 (100)	0.80 $\pm$ 0.16 (100)	3.43 $\pm$ 0.66 (100)	3.3 $\pm$ 0.1 ^a
25-15	0.19 $\pm$ 0.10 (7)	0.19 $\pm$ 0.02 (24)	0.38 $\pm$ 0.12 (11)	1.0 $\pm$ 0.4 ^c
15-25	0.73 $\pm$ 0.51 (28)	0.21 $\pm$ 0.14 (26)	0.94 $\pm$ 0.65 (27)	3.5 $\pm$ 0.2 ^a
15-15	0.12 $\pm$ 0.05 (5)	0.06 $\pm$ 0.02 (8)	0.19 $\pm$ 0.07 (5)	2.0 $\pm$ 0.1 ^b

†, Growth temperature; ‡, incubation temperature. Values are expressed as mean $\pm$ S.D. of three individual experiments. The numbers in parenthesis are the relative values to those of control (25°C-25°C). Figures followed by a different letter are significantly different at the 5% level.

kDa in soluble proteins, some thylakoid proteins with a molecular size of about 25~32 kDa in insoluble proteins, and several bands in soluble and insoluble proteins, especially those with a molecular weight higher than LSU, were present at lower intensities. The intensity of the band with a molecular size of about 16 kDa in insoluble proteins was even higher than the same plants incubated at 25°C (lanes 1, 2, 5 and 6 in Fig. 1). At low incubation temperature, distribution of radioactivity in soluble and insoluble proteins in the stressed plants was similar to that in the control plants, except lower intensities of SSU of RuBPC with the molecular size of 15 kDa and several bands of proteins with a molecular weight lower than LSU (lanes 2, 4, 6 and 8 in Fig. 1). However, synthesis of LSU, SSU, and several bands apparently recovered in the stressed plants when incubated at 25°C (lanes 3 and 7 in Fig. 1).

It is reported that protein synthesis is markedly inhibited by low temperature below 18~20°C in rice^{7,18)}. Previous experiments showed that rice plants exposed to low temperature had a higher soluble nitrogen content, or free amino acids, as compared with control plants⁸⁾, and that the ATP level of rice plants hardly changed at a growth temperature between 6 to 26°C⁷⁾. Therefore, it is unlikely that the inhibition of protein synthesis in developing leaves at a low temperature is caused by reduction of photosynthesis or shortage of carbohydrate and amino acids translocated from the other parts of the plants. It is suggested that protein synthesis itself is inhibited by low temperature.

In the present work, a low temperature of 15°C not only reduced the rate of protein synthesis, but also drastically changed the distribution of synthesized proteins. Synthesis of RuBPC, several thylakoid proteins and other unidentified proteins was severely suppressed, while synthesis of some proteins was induced by low temperature. Hahn and Walbot⁵⁾ reported a similar result, that synthesis of RuBPC was drastically reduced, and some polypeptides were induced after cold treatment in rice. In addition, genes for proteins induced under low temperature were investigated and isolated in rice plant^{1,11,13)}. Alterations in protein synthesis by chilling stress are also reported in chilling-sensitive plants, such as tomato³⁾ and mung bean⁹⁾.

3. Effect of low temperature on synthesis of RuBPC subunits

RuBPC synthesis was investigated by measuring the radioactivity of immunoprecipitated RuBPC proteins in the control and the stressed plants incubated at 15°C and 25°C. Incorporation of radioactivity into LSU and SSU was measured after separation of immunoprecipitated RuBPC protein subunits by SDS-PAGE. When the control plants were incubated at 15°C, incorporation of [^{35}S]methionine into RuBPC subunits was drastically reduced to 11% of the same plants incubated at 25°C (Table 3). The incorporation rate into RuBPC in the stressed plants incubated at 15°C was only 5% of the control plants measured at 25°C, whereas the rate increased to 27% when incubated at 25°C. Decrease of incorporation rate into LSU by low incubation temperature was much larger, and that by low

Fig. 2. Densitometric tracings (A-D) and radioactivity tracings (E-H) from gels of immunoprecipitated RuBPC subunits.

A and E, 25°C (growth temperature)-25°C (incubation temperature); B and F, 25°C-15°C; C and G, 15°C-25°C; D and H, 15°C-15°C. L and S indicate large and small subunits of RuBPC, respectively.

growth temperature was slightly smaller, as compared with SSU. Thus, the ratio of incorporation rate into LSU to SSU (LSU/SSU) in the control plants was 3.3 at 25°C, however, it decreased to 1.0 when incubated at 15°C. Although LSU/SSU in the stressed plants was 2.0 incubated at 15°C, it came up to 3.5 when measured at 25°C.

The intensity and radioactivity of LSU and SSU on the gels was scanned after immunoprecipitation and SDS-PAGE, and the densitometric and the radioactivity tracings are shown in Fig. 2. When the control plants were subjected to a low temperature of 15°C, the relative area of radioactivity of SSU to LSU greatly increased as compared with the same plants incubated at 25°C (E and F in Fig. 2), while the ratio of amount of SSU to LSU was scarcely changed (A and B in Fig. 2). No substantial difference was observed in radioactivity tracings between the control plants measured at 25°C and the stressed plants (E, G and H in Fig. 2), although tendency of lower relative area of amount of SSU to LSU was observed in the stressed plants than the control ones (A, C and D in Fig. 2).

When the temperature responses in synthe-

sis of RuBPC subunits were compared with those of soluble proteins, a decrease in the incorporation rate of radioactivity into RuBPC subunits, both by low-growth and low-incubation temperatures, was larger than that for soluble proteins (Tables 2 and 3). The result suggests that synthesis of RuBPC is specifically susceptible to low temperature. The present work further investigated synthesis of individual subunits of RuBPC, in order to analyze the suppression of RuBPC synthesis due to low temperature. Evidence was obtained that suppression in synthesis of LSU was greater than that of SSU at low incubation temperature, indicating that the synthesis of LSU is more sensitive to low temperature than that of SSU. In addition, it is also suggested that coordination of LSU and SSU synthesis is lost under low temperature. Hahn and Walbot⁵⁾ observed that synthesis of LSU was inhibited more than that of SSU at the beginning of cold treatment, although synthesis of SSU was more strongly suppressed than that of LSU after 7 days at low temperature.

Genes for LSU and SSU are encoded by chloroplast and nuclear DNA, respectively, and coordinated synthesis and assembly of the protein complex is accomplished by cellular or environmental signals¹⁷⁾. The results suggest that the synthesis of RuBPC proteins, especially in the part associated with chloroplasts, is specifically susceptible to low temperature, and this could be one of the factors retarding photosynthesis and development of leaves in rice plants grown under low temperature. Further investigations are needed on regulatory mechanisms of protein synthesis in developing leaves of rice under low temperature.

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