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Photosynthetic Control Factors in a Single Leaf of Sweet Potato, *Ipomoea batatas* Lam.*

3. Estimation of *in vivo* rubisco activity from the CO_2 exchange rate of a peeled leaf

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Abstract : Rubisco (ribulose 1,5-bisphosphate carboxylase/oxygenase) activity is the prime determinant for CO_2 exchang rate (CER) at the beginning of the photosynthetic reaction, and hence the rubisco activity in a leaf can be deduced from the initial rise of CER. The activity of rubisco is also regulated by two main internal factors, the activation state of rubisco and the availability of RuBP (ribulose 1,5-bisphosphate). A peeled leaf (*Ipomoea batatas*, cv. Koganesengan) without stomatal resistance was preilluminated in PAR of 900 μmol m^{-2} s^{-1} at CO_2 concentration ($[CO_2]$) of 20, 60 or 350 μmol mol^{-1}. Directly after the dark interruption following preillumination, the response of CER of a peeled leaf (CER_{P1}) to reillumination (PAR of 900 μmol m^{-2} s^{-1}) was measured at $[CO_2]$ of 350 μmol mol^{-1}. The dark time affected the response of CER_{P1}. The light-activated state of rubisco, which was deduced from the response of the initial rise of CER_{P1} (IR-CER_{P1}), could remain constant for at least 5 min in the dark. The leaves preillluminated at different $[CO_2]$ levels had no or little difference in IR-CER_{P1} which was determined after the dark interruption of 2.5 min. This is an evidence that rubisco in the intact mesophyll tissues was substantially light-activated even at such a low $[CO_2]$ level as 20 or 60 μmolmol^{-1} and the rubisco activity was primarily determined by the activation state of rubisco but was independent of the RuBP pool level. IR-CER_{P1} can be proposed as an indicator for the rubisco activation state *in vivo* or the rubisco activity unrestricted by RuBP availability.
Key words : CO_2 exchange rate, *Ipomoea batatas* Lam., Leaf epidermis peeling, Rubisco activity.

カンショにおける個葉光合成速度の支配要因の解明　第3報　表皮剥離葉の炭酸ガス交換速度からの生体内ルビスコ活性の推定：窪田文武・名田和義・縣　和一（九州大学農学部）

要　旨：ルビスコ活性は，炭酸固定反応開始時のガス交換速度（CER）の決定主要因であり，CERの初期勾配から葉内のルビスコ活性を推定できる．また，ルビスコ活性はルビスコの活性化状態と基質RuBP（リブロース1,5ビスリン酸）量の二つの内的要因に制御される．ガス交換の及ぼす気孔の影響を取り除いた表皮剥離葉（カンショ品種コガネセンガン）を異なるCO_2濃度条件（20，60および350 μmol mol^{-1}）で光照射（前処理）し，暗処理をはさんで，光再照射（900 μmol m^{-2} s^{-1}）に対する剥離葉のCER（CER_{P1}）の反応をCO_2濃度350 μmol mol^{-1}条件下で測定した．CER_{P1}反応に暗処理時間の影響が認められた．CER_{P1}の初期勾配（IR-CER_{P1}）から，ルビスコの光活性化状態は暗所で少なくとも5分間維持できるものと判断された．暗処理を2.5分間とした場合のIR-CER_{P1}には，前処理におけるCO_2濃度の差の影響は認められなかった．これは，20および60 μmol mol^{-1}のような低いCO_2濃度下においても葉内ルビスコの光活性化が可能であることを示すとともに，反応開始時におけるルビスコ活性（IR-CER_{P1}）は主にルビスコの活性化状態によって決定されており，RuBPの蓄積量の影響を受けないことを示す．IR-CER_{P1}から生体内のルビスコの活性化状態，すなわち，RuBP供給に制限されない状態でのルビスコ活性を推定することが可能である．
キーワード：ガス交換速度，カンショ，葉表皮剥離，ルビスコ活性．

Rubisco (ribulose 1,5-bisphosphate carboxylase/oxygenase) catalyzes the initial reaction of CO_2 fixation with both substrates, CO_2 and RuBP (ribulose 1,5-bisphosphate), and hence rubisco can be regarded as the key enzyme for photosynthesis in C_3 plants[18,26]. CO_2 exchange rate (CER) is controlled by the complicated biochemical functions of rubisco and the other enzymes in the C_3 cycle, but just upon the initiation of CO_2 fixation, CER is primarily regulated by the activity of rubisco, which is also governed by two main internal factors, the rubisco activation state and the RuBP availability in chloroplasts.

Rubisco activity is usually assayed *in vitro* using a biochemical technique with extracts from leaf tissues. However, the rubisco in extracts is never stable in activity and readily

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inactivated depending on the assay condition. To determine the natural activity of rubisco and the other enzymes related to photosynthesis, the assay procedure has been improved[1].

Gas exchange analysis is also used as a nondestructive method for assaying rubisco activity. The rubisco activity in intact leaves of the C_3 plants is estimated from the initial slope of the CO_2 response curve of CER[4,5,10,11,22,27]. However, with these methods the CO_2 exchange is often strongly affected by stomatal resistance depending on environmental conditions, and this causes to make CER response unclear and variable.

A peeled leaf of *I. batatas* was used here as a stomatal resistance-free material. The activation state of rubisco and RuBP pool size in the chloroplasts were modulated by preconditioning a peeled leaf, and its effects on the *in vivo* activity of rubisco were deduced from the initial rise of CER in the peeled leaf upon illumination.

Material and Methods

Meterial

As an experimental material, a cultivar (cv. Koganesengan) of sweet potato, *Ipomoea batatas* Lam., was used. A young shoot was transplanted on April 20 1992 into a 10 L pot containing sandy soil with chemical components (N, P, K ; 1.6g/pot each), and grown for about two months in a glasshouse set up in the experimentas field of Kyushu University. Fully expanded young leaves were used for treatments and measurements.

CER measurement, and modulation of the rubisco activation state and RuBP pool size in a peeled leaf

Using a technique described in previous papers[16,17], the abaxial epidermis was peeled from a leaf. The stomatal density of the abaxial epidermis was about 250/mm^2, roughly 5-fold that of the adaxial side. The adaxial surface was covered with a transparent film to prevent the gas exchange. The leaf was enclosed in an assimilation chamber (SPB-H3, ADC, Britain) and CER of the peeled abaxial surface (CER_{P1}) was measured. Leaf area used for the measurement was 6.25 cm^2.

The rubisco activation state and RuBP pool size in intact tissues were modulated by placing a peeled leaf under different combinations of light, dark and CO_2 concentration ($[CO_2]$) levels. The treatment procedure is outlined in Fig. 1 (step A, B and C).

At the step A, as a rubisco activation treatment, a peeled leaf set in the assimilation chamber was illuminated for 20 to 30 min, during which $[CO_2]$ was adjusted to three levels, 350 (the atmospheric level), 60 (a CO_2 compensation level) or 20 μmol mol^{-1} (a level at which CO_2 assimilation almost stopped). The illumination (PAR, photosynthetic active radiation) was 900 μmol m^{-2} s^{-1}, which was a saturation light intensity for the photosynthesis measured in $[CO_2]$ of 350 μmol mol^{-1} at a leaf temperature of 30°C.

Then, at the step B, the illumination treatment was interrupted by the dark of different periods, during which $[CO_2]$ in the assimilation chamber was 350 μmol mol^{-1}.

Finally, at the step C, CER_{P1} was measured at 10 s intervals at $[CO_2]$ of 350 μmol mol^{-1} immediately after initiation of reillumination (PAR of 900 μmol m^{-2} s^{-1}), in order to determine the initial rise of CER_{P1} (IR-CER_{P1}) and the time course of CER_{P1}. IR-CER_{P1} was given here as the initial rise presented within the 20 s after reillumination.

During the treatments and measurements, the relative humidity (RH) in the assimilation chamber was maintained at about 90% to

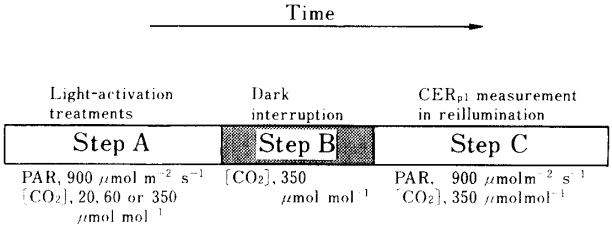

Fig. 1. Outline of light/dark/light transient treatments. At step A, a peeled leaf, which was mounted in the assimilation chamber, was held in PAR of 900 μmol m^{-2} s^{-1} at different $[CO_2]$ levels of 20 to 350 μmol mol^{-1}. Then at step B, the illumination treatment was interrupted by the dark for 1 to 180 min, during which $[CO_2]$ in the chamber was adjusted to 350 μmol mol^{-1}. Finally at step C, immediatelly after reillumination (PAR of 900 μmol m^{-2} s^{-1}), CER_{P1} was measured at 10 s intervals at $[CO_2]$ of 350 μmol mol^{-1}. Peeled leaf, the abaxial epidermis was peeled ; CER_{P1}, CO_2 exchange rate on the abaxial surface of a peeled leaf

prevent the photosynthetic apparatus of the peeled leaf from suffering desiccation damages. Velocity of the air fed to the chamber was 400 mL min^{-1} per 6.25 cm^2 leaf area. Light was provided through the transparent film cover to the adaxial surface of the leaf by a slide projector (HILUX-HR, Rikagaku, Japan) with an 1 KW halogen lamp.Adjustment of $[CO_2]$ level was made by passing the atmospheric air through a tube filled with soda lime as a CO_2 absorbent.

Results and Discussion

Effect of epidermis peeling on the light-photosynthetic response

First the effects of epidermis peeling on CER and leaf conductance (Gl) were examined. A leaf with or without the abaxial epidermis was held in the dark for 120 min in advance, directly after this CER and Gl were periodically measured on the abaxial side of the leaf in PAR of 900 μmol m^{-2} s^{-1} at $[CO_2]$ of 350 μmol mol^{-1}.

The time courses of CER (or CER_{P1}) and Gl are shown in Fig. 2. CER of an unpeeled leaf had a curvilinear increase within 1 min after initiation of illumination, but the following increase was very slight ; CER did not yet reach 8.0 μmol m^{-2} s^{-1} after 30 min. On the other hand, the light-sensitivity of photosynthesis was greatly enhanced by peeling the epidermis ; CER_{P1} had a quick increase responding to illumination, then reached a maximal steady state (about 10μmol m^{-2} s^{-1}) within 4 min. Rubisco in the leaf is expected to get a light-activated state during this time.

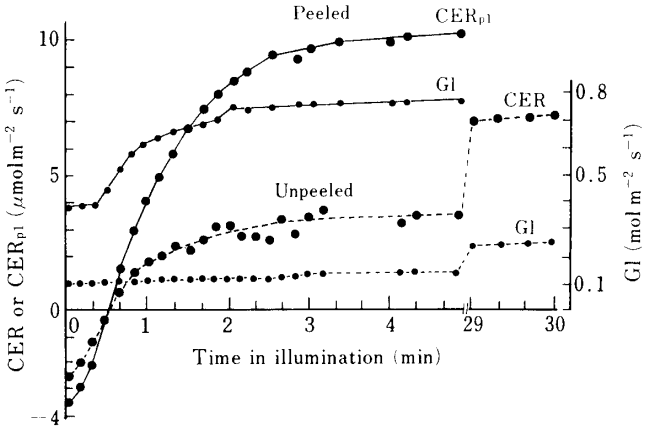

Fig. 2. Differences in the time course of CER (or CER_{P1}) and Gl between peeled and unpeeled leaves. The leaves were held in the dark for 120 min in advance, then both parameters on the abaxial side were measured at short intervals in PAR of 900 μmol m^{-2} s^{-1} at $[CO_2]$ of 350 μmol mol^{-1}. Leaf temperature was 30.3 to 30.7° C. Real line, peeled leaf ; Broken line, unpeeled leaf.

Through the predark treatment of 120 min, Gl of the unpeeled leaf reduced to 0.12 mol m^{-2} s^{-1} by stomatal closure. The slow light-response of stomatal opening seems to be a major cause of the slow increase in CER, while Gl in the peeled leaf was above 0.46 mol m^{-2} s^{-1}, allowing a sufficient gas exchange.

Kobza and Edwards[12] have noted that rubisco in *Triticum aestivum* L. was light-activated within 1 min, but the time required for reaching the steady state of photosynthesis was much longer, several to several tens of minutes. Of many factors regulating photosynthesis, stomatal resistance is often taken up as a major cause of enlarging the gap between CER and rubisco activity in a leaf. To remove the influence of stomatal movement on CER is a prerequisite for estimating the rubisco activity by gas exchange method.

Kirschbaum and Pearcy[10], and Sassenrath-Cole and Pearcy[22] have tried to divide the time course response of CER to light in the C_3 plants, *Alocasia macrorrhiza* (L.) G. Don and *Glycin max* L., into three phases, the initial increase, and the fast and slow induction phases. They have discussed the fast and slow phases by taking advantage of the difference in reaction speed to illumination between the fast phase components (RuBP regeneration capacity) and the slow phase components (rubisco activation and stomatal resistance). However, it is difficult to remove completely the influence of stomatal resistance on CER and, depending on the treatment conditions, the initial rise in CER is strongly restricted by the existence of stomatal resistance.

Stomata work as a variable resistance to the gas exchange from leaf surface to intercellular air spaces. In addition, the mesophyll resistance also plays a significant role in regulating the CO_2 diffusion from intercellular air spaces to carboxylation sites in chloroplasts[6]. However, in a constant ambient $[CO_2]$ environment, the variability of mesophyll resistance is much smaller than that of stomatal resistance. Therefore CER_{P1} can be recognized as a

stomatal resistance-free CER which is more closely associated with the CO_2 fixation capacity in mesophyll cells.

Effect of dark interruption periods on the response of CER_{P1} to reillumination

A peeled leaf in the assimilation chamber was treated in the order of the step A, B and C shown in Fig. 1. The leaf was first fully light-activated in PAR of 900 μmol m^{-2} s^{-1} at $[CO_2]$ of 350 μmol mol^{-1} for 20 min (step A). This illumination effect was then interrupted by the dark periods ranging from 1 to 180 min (step B). The leaf was then reilluminated in PAR of 900 μmol m^{-2} s^{-1} at $[CO_2]$ of 350 μmol mol^{-1}, and CER_{P1} was measured at 10 s intervals directly after reillumination initiation (step C). The same portion of the same leaf was used in series here. The total time of illumination on the single leaf was about 150 min, during which the photosynthesizing capacity of the leaf was not reduced.

Effect of periods of the dark interruption treatment on CER_{P1} is shown in Fig. 3. The

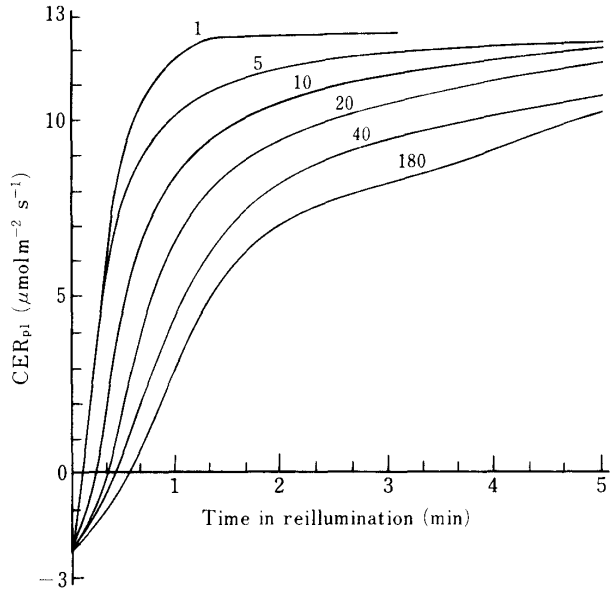

Fig. 3. Effect of dark interruption periods on the response of CER_{P1} to reillumination. A peeled leaf was light-activated in PAR of 900 μmol m^{-2} s^{-1} at $[CO_2]$ of 350 μmol mol^{-1} for 20 min (step A). Then the leaf was held in the dark for 1, 5, 10, 20, 40 or 180 min (step B). CER_{P1} was measured at 10 s intervals in PAR of 900 μmol m^{-2} s^{-1} at $[CO_2]$ of 350 μmol mol^{-1} (step C). Leaf temperature was 28.3 to 29.8°C through these steps. See Fig. 1 for step A, B and C.

time course of CER_{P1} varied from hyperbolical to sigmoidal curve with predark treatment time. According to the reports referred above, the time course response of CER to light has the two inflection points, by which the responce was devided into the three phases. However, we detected no inflection point between the initial increase phase and fast induction phase. The $[CO_2]$ monitoring interval (10 s) used in our experiment seems a little too long for detecting the inflection point in the quick initial response of CO_2 exchange. Nevertheless, as shown in Fig. 3, there were clear treatment differences both in IR-CER_{P1} and in CER_{P1} time course. A decline of IR-CER_{P1} indicates that the rubisco activity reduced with predark treatment time. Rubisco functions not only as a carboxylase but also as an oxygenase[7,8,9,26], and hence the rubisco activity deduced from IR-CER_{P1} is regarded as a balanced activity between both enzymatic functions.

It has been described that rubisco in *Triticum aestivum* L. leaf was able to maintain a relatively high activation state for 5 min in the dark[12]. In our results (Fig. 3), when the dark treatment period was short, 1 or 5 min, CER_{P1} had a steep initial rise, which indicates that rubisco in a leaf of *I. batatas* also maintained an activated state for at least 5 min in the dark.

A reduced RuBP pool in chloroplasts becomes a cause of decreasing rubisco activity. From the reported facts that RuBP pool in chloroplasts quickly reduced in the dark[19,22,23], we expected that there was some difference in IR-CER_{P1} between the leaves subjected to 1 min and 5 min predark treatments, but there was no difference in it as mentioned above. RuBP pool reduction does not seem to regulate strongly the initial photosynthetic rate here.

When the predark time exceeded 5 min the time-course curve of CER_{P1} gradually became sigmoidal; having a gentle initial slope. The depression in IR-CER_{P1} shown here is considered to depend on inactivation of rubisco and/or RuBP deficiency. Furthermore when the predark was 180 min, the curve presented a stepwise increase. The patternal variation in CER_{P1} time course may be associated with a more complicated process. The light activation processes of rubisco have been discussesd in

connection with both rubisco activases[2,3,20,25] and activation inhibitors[9,13,14], and several enzymes other than rubisco in the C_3 cycle is known to have different light-dependent properties in the activation process[18,22,26]. It may be, thus, suggested that the enzymes which have been once inactivated in the dark can not be uniformly reactivated within a short time after reillumination. The unbalanced relays from substrate production to consumption in the C_3 cycle might have caused the disturbance of CER_{P1} time course pattern.

IR-CER_{P1} in the leaf light-activated at different $[CO_2]$ levels

We tried to modulate both the rubisco activation state and RuBP availability in chloroplasts by illuminating a peeled leaf at different $[CO_2]$ levels. The effect of the modulations on rubisco activity was deduced from IR-CER_{P1}.

First, a peeled leaf in the assimilation chamber was held in the dark for 10 min to reduce the RuBP pool in chloroplasts. Thereafter the leaf was subjected to each step shown in Fig. 1. At the step A, the leaf was exposed to PAR of 900 μmol m^{-2} s^{-1} at $[CO_2]$ of 20, 60 or 350 μmol mol^{-1} for 30 min. The dark interruption time at the step B was 2.5 min, which was the time required for adjustment of $[CO_2]$ level in the chamber from 20 or 60 to 350 μmol mol^{-1}. During this time the activation state of rubisco can be maintained constant, as mentioned above. At the step C, CER_{P1} was measured by time-course in PAR of 900 μmol m^{-2} s^{-1} at $[CO_2]$ of 350 μmol mol^{-1}. The treatments and measurements were made in series using the same peeled portion of the same leaf.

Time courses of CER_{P1} are shown in Fig. 4. When the leaf was preilluminated at $[CO_2]$ of 350 μmol mol^{-1}, CER_{P1} had a sharp initial slope upon reillumination, then reached a maximal level within 1 min. Also a similar sharp response was detected in the initial phase of CER_{P1} in the leaf which had been preilluminated at a very lower $[CO_2]$ level (60 or 20 μmol mol^{-1}). Although it has been reported that rubisco in *Phaseolus vulgaris* L. was highly activated at a low intercellular $[CO_2]$ such as 80 μmol mol^{-1} at the *in vitro* level[21], our results strongly indicate that rubisco in a leaf of *I. batatas* could be light-activated even at lower $[CO_2]$ levels than 80 μmol mol^{-1}.

RuBP in the chloroplasts is noted to

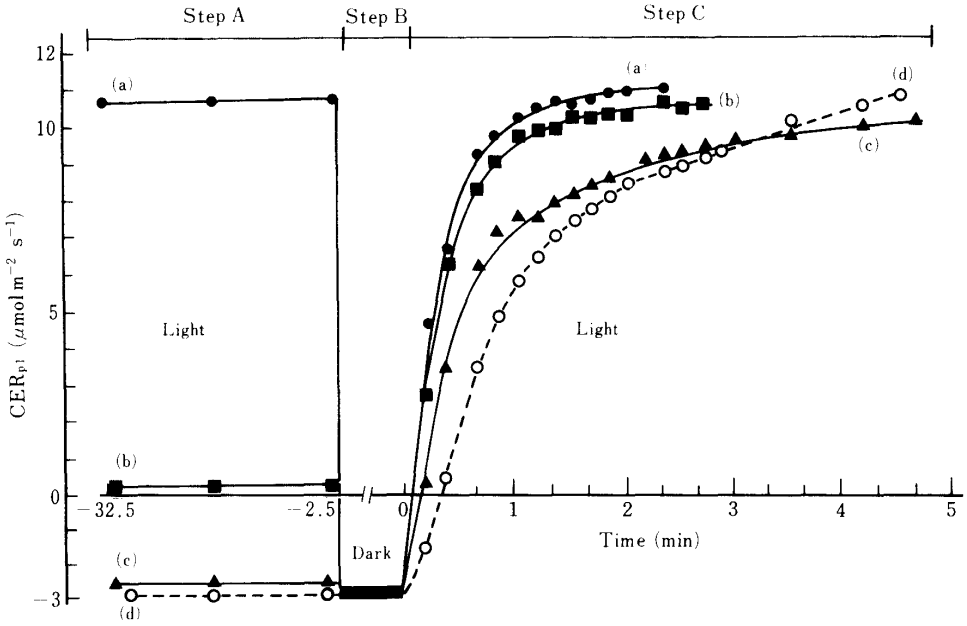

Fig. 4. Effect of preillumination at different $[CO_2]$ levels on the time course of CER_{P1}. A peeled leaf was held in PAR of 900 μmol m^{-2} s^{-1} at $[CO_2]$ level of 350 (a), 60 (b) or 20 (c) μmol mol^{-1} for 30 min (step A). The leaf (d) was held in the dark for 30 min. After a dark interruption of 2.5 min (step B), CER_{P1} was measured at 10 s intervals in PAR of 900 μmol m^{-2} s^{-1} at $[CO_2]$ of 350 μmol mol^{-1} (step C). Leaf temperature was 29.2 to 30.0°C through these treatment steps. See Fig. 1 for step A, B and C.

increase in volume when a leaf is illuminated at lower $[CO_2]$ levels than the atmospheric standard because the carboxylation reaction is restricted[5,19,23]. However, if a leaf is placed at an extremely low $[CO_2]$ level, such as 20 or 60 μmol mol^{-1}, both CO_2 uptake (the carboxylase function of rubisco) and RuBP regeneration are almost stopped, and RuBP, even if somewhat pooled, can be consumed as a substrate for photorespiration (the oxygenase function of rubisco). Pools of RuBP and the other substrates in the RuBP regeneration cycle are presumed to drop to bottom levels by this treatment.

However, this treatment had no or little effect on IR-CER_{P1}, as shown in Fig. 4. This fact indicates that under the measurement conditions given here, IR-CER_{P1} (or rubisco activity) is primarily restricted by activation state of the rubisco but is independent of accumulation levels of RuBP. That is to say, CO_2 fixation is able to begin at a suffciently high rate using a residual pool of RuBP. Hence IR-CER_{P1} can be proposed as an indicator of rubisco activation state or rubisco activity without restriction of RuBP supply. Also the effect of pretreatments on rubisco activation state can be deduced from the response of IR-CER_{P1}.

In the leaf preconditioned at $[CO_2]$ of 20 μmol mol^{-1}, the angle of IR-CER_{P1} began to reduce about 45 s after reillumination initiation (Fig. 4, (c)). This photosynthetic retardation may be considered to depend on a delay of RuBP regeneration.

When the carboxylation of a leaf is stopped in a strong light by low $[CO_2]$ conditions, the photochemically produced energy is expected to accumulate excessively in the chloroplasts and have adverse effects on the photosynthetic apparatus. However, Sharkey *et al.*[24] have noted that the capacity for electron transport was matched to the capacity for the other processes required by photosynthesis so that the electron transport rate could be adequately regulated under low $[CO_2]$ conditions. Also the photorespiration cycle of C_3 plants can function as a sink of energy[15]. In our experiment, a steep IR-CER_{P1} was observed in a leaf preconditioned at such a low $[CO_2]$ level as 20 or 60 μmol mol^{-1}, and CER_{P1} smoothly came up to the maximal state in reillumination (Fig. 4). This may prove that the photosynthetic function of *I. batatas* leaf was not damaged by the treatments.

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