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Sequestration of Orthophosphate by D(+)-Mannose Feeding Increases Nonphotochemical Quenchings in Chinese Cabbage Leaves

Park, Youn-Il, Kye Hong Suh* and Young-Nam Hong
(Department of Biology, Seoul National University, Seoul and
**Department of Biology, Taegu University, Taegu)*

Mannose 처리된 배추 잎의 무기인산 감소에 따른 비광화학성 소산의 증가

朴 淵 一·徐 桂 弘*·洪 英 男
(서울대학교 생물학과, *대구대학교 생물학과)

ABSTRACT

Limitation of photosynthesis in detached Chinese cabbage (*Brassica campestris* L.) leaves was induced by feeding of mannose (25 mM) for 12 h in the light, and changes in the basic thylakoid functions under this condition were investigated. The acid soluble phosphate content and CO_2 uptake rate was decreased by 66% and 67%, respectively. However, the starch content was increased by 24% compared to those of controls. From the fast induction curves of chlorophyll fluorescence, dark level fluorescence (F_O) slightly increased while intermediate plateau fluorescence level (F_I) to peak level fluorescence (F_P) transient was significantly decreased with a slight decrease in the F_O-to-F_I transient. This data means that reduction of secondary electron acceptor of PSII (Q_B) might be more severely inhibited than that of primary electron acceptor of PSII (Q_A) by decrease in phosphate level. The strong decline of $(F_v)_m//F_m$ ratio suggests that efficiency of excitation energy capture by PSII was decreased markedly. The quenching of F_O (qO), an indicator of state transition, was also occurred over the slow induction kinetics of chlorophyll fluorescence. From quenching analysis, fluorescence was dominantly quenched by nonphotochemical quenchings (qE+qT). These results showed that the capture and transfer efficiency of excitation energy to PSII reaction center in thylakoid was decreased with the decline of leaf phosphate level, and that the state transition was occurred during the induction of photosynthesis under these conditions.

INTRODUCTION

Recent researches showed that the maximum rate of photosynthesis can be restricted by the rate of end-product synthesis through selective inhibition of sucrose synthesis (Harris *et al.*, 1983; Quick *et al.*, 1989). These previous studies confirm the view that the flux of phosphate can limit photosynthesis (Herold *et al.*, 1976; Rao and Terry, 1989; Sharkey, 1985; Sharkey *et al.*, 1986). However, it remains unclear how the flux of phosphate ultimately feeds back to the function of thylakoid membrane. Only a few contradictory results were reported to the tuning response of thylakoid membranes to phosphate concentrations in the intact chloroplast (Gierish and

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Robinson, 1987; Heineke *et al.*, 1989).

The basic function of thylakoid (i.e., the provision of $NADPH+H^+$, ATP and the modification of stromal environment) may be fed back by redox equilibria or via the proton gradient across the thylakoid membrane. It is well known that both redox feedback to PSII (and hence affects photochemical quenching) and acidification of the inner-thylakoid membrane (revealed in the change of non-photochemical quenching) affect the characteristic changes in chlorophyll fluorescence emitted from PSII (Briantais *et al.*, 1986; Krause and Weis, 1984, 1991). Thus, fluorescence analysis provides an excellent tool to study feedback during assimilation *in vivo* by metabolic factors (Horton, 1987; Weis and Lechtenberg, 1989) or environmental factors (Baker *et al.*, 1989; Schreiber and Bilger, 1987). It is obvious that various quenching mechanisms determine the chlorophyll fluorescence yield *in vivo*, allowing assessment of the relative overall photosynthetic rate under induction of photosynthesis. Major quenching components are photochemical quenching (qQ), due to photochemical energy conversion at PSII reaction centers, and the non-photochemical quenchings (qNP) which consist mainly of energy-dependent quenching (qE) due to an increased rate of radiationless deexcitation upon energization of the thylakoid membrane (Krause and Weis, 1984). qQ is hence an indicator of the changes in photochemical efficiency due to redox feedback from the electron transfer chain and qNP is an indicative of regulation of the dissipatation of excitation energy in the thylakoid. Actually, it was reported that the redox state change by anaerobiosis (Satoh and Fork, 1982) or CO_2 depletion (Bilger and Schreiber, 1986) markedly induce the alteration of the chlorophyll fluorescence kinetics. Changes in phosphorylation potential by sequestering (Harris *et al.*, 1983, 1986) or lowering P_i (Abadia *et al.*, 1987) affected these kinetics, also.

In the present study, we investigated the effect of lowered-P_i on the basic function of thylakoid membrane in the Chinese cabbage leaves, using room temperature chlorophyll fluorescence which is now an accepted non-intrusive probe of photosynthetic processes *in vivo* and *in vitro* (Krause and Weis, 1991).

MATERIALS AND METHODS

Plant material. Chinese cabbage (*Brassica campestris* L. cv. Seoul) seeds surface-sterilized with 1% sodium hypochlorite for 10 min were sown in a polypropylene container (11.5×11.5×10 cm) with a glass cover containing vermiculate (about 10 g) fertilized with 80 m*l* of half-strength Hoagland solution (containing 0.5 mM KH_2PO_4). 20 seedlings per container were grown in a growth cabinet at a constant light/dark temperature of 25℃ with 80 $\mu Em^{-2}s^{-1}$ photon fluence rates from day light tube (General Electric, U.S.A.) at canopy levels and a light/dark cycle of 15/9 h. The seedlings were fertilized daily with 20 m*l* of the nutrient solution. After 15 day growth, primary leaves with 2 cm petiole from the leaf basal were removed and used for all experiments. Detached leaves were floated in 25 mM D-mannose and subsequently illuminated for 12 h in growth cabinet.

Estimation of acid soluble leaf phosphorous concentration. Acid soluble phosphorous (P_i) content in leaf was estimated by developing the blue phosphomolybdate color and reading its intensity by colorimeter (Terry and Ulrich, 1973).

Estimation of CO_2 uptake rates. Rates of CO_2 uptake from a single detached leaf were measured using an open-gas exchange system with a modified Parkinson leaf chamber (Type B, ADC Co., U.K.). Illumination was provided by actinic light (KL1500 lamp, equipped with Schott RG 630, 150W halogen lamp, Osram xenophot HLX) through fiberoptic systems used in measurement of chlorophyll fluorescence. Light intensity measured by quantum sensor (Delta-T devices Ltd, QS 8537, U.K.) was 1200 $\mu molm^{-2}s^{-1}$. Atmospheric air from outdoors (340-360 vpm) was water-saturated and then passed through the chamber at a rate of 200 $ml \cdot min^{-1}$ with Air Supply Unit (ADC Co., U.K.). Leaf temperature sensed by means of a T-type thermistor was nearly constant at 28± 1℃ during experiments. The difference in partial pressure of CO_2 in the ingoing and outgoing air streams was measured with Infra Red Gas Analyzer (LCA2, ADC Co., U.K.). The outputs of all sensors were continuosly logged in data logger (Delta T devices, U.K.). A calculation of CO_2 uptake rate per unit area of leaf was made according to Suh and Kim (1981).

Estimation of starch and sucrose contents. Starch and sucrose were extracted according to Wilson and Lucas (1978) with following modifications. 5 leaves were placed into a test tube. Then 5 m*l* of 80% ethanol added, and the tube was placed in a 100℃ bath for 1 min to kill tissue. The ethanol was removed with a Pasteur pipette and collected in a second tube. An additional 3 m*l* of 80% ethanol was added to the detached leaves. The tube was then placed in a 65± 1℃ water bath to extract the ethanol-soluble sugars. This procedure was repeated until all the chlorophyll was removed from detached leaves (usually 2 repetitions sufficed). 2 m*l* of DW was added to test tube, and the tube was placed in a

boiling water for 1 h to gelatinize the starch. After the tubes were cooled with tap water, the DW was decanted. 2 m*l* of 15 mM sodium acetate buffer (pH 4.5) containing 0.5% (w/v) amyloglucosidase (Sigma) was added and the tubes incubated at 55℃ for 18 h. The detached leaf was stained with iodine-potassium iodide and examined under a light microscope to check for complete digestion of starch. The released glucose was analyzed with glucose oxidase kit system (Sigma 510A). 2 m*l* aliquot of ethanol extract was placed in a test tube and the Chl was removed with 2 m*l* chloroform. 1.5 m*l*-aliquot of the upper clear phase was used for sucrose analysis. Sucrose concentration was determined using the resorcinol test using sucrose standard after other ketose sugars were destroyed with 1.0 N NaOH in boiling water for 10 min (Huber, 1983).

Estimation of photosynthetic pigments. Chlorophyll and total carotenoid of leaf were extracted with dimethylsulfoxide (DMSO) (Hiscox and Israelstham, 1979). After incubation of 5 leaves with 5 m*l* of DMSO for 30 min at 65℃, Chl a, Chl b, Chl a/b and total carotenoid contents were measured according to Lichtenthaller (1987).

Estimation of chlorophyll fluorescence at room temperature. Measurements of modulated chlorophyll fluorescence emission from the upper surface of the detached leaves were made using a pulse amplitude modulation fluorimeter (PAM 101, 102, and 103, H. Walz, Effeltrich, F.R.G.). Dark-adapted leaf at least 1 h at 25℃ was placed in a leaf holder with a small volume of gas container and exposed to a stream of water-saturated air (200 $ml \cdot min^{-1}$) through the lower surface of detached leaf during induction of chlorophyll fluorescence. For the recordings of fast fluorescence changes upon a dark-light transitions were conducted with IBM compatable computer mounted with interface board. The interface board contains a 12 bit D/A and seven A/D converters (PCL 711, Advantech Co., Taiwan). For the slow fluorescence induction kinetics, dark-adapted leaves (at least 1 h at 25℃) were initially exposed to the weak modulated measuring beam (appox. 0.05 $\mu Em^{-2}s^{-1}$), followed by exposure to a continuous actinic light (84 $\mu Em^{-2}s^{-1}$). A 800 ms pulse of high intensity white light (3400 $\mu Em^{-2}s^{-1}$) was used to produce a transient closure of the PSII photochemical reaction centers. The traces were registered on recorders. Fluorescence quenching coefficients were determined essentially as described (Schreiber, 1986) except that quenchings of F_0 was taken into account (Bilger and Schreiber, 1986).

RESULTS AND DISCUSSION

Effects on phosphate content, CO_2 uptake rate, sucrose and starch contents. Mannose has been usually used as a phosphate sequestering agent as expected (Harris *et al.*, 1983, 1986; Herrold *et al.*, 1976; Walker, 1981; Wilson and Lucas, 1987), and as confirmed (Sharkey and Vanderveer, 1989). Whether mannose feeding can induce the limitation of photosynthesis in Chinese cabbage leaves through the changes in phosphate level were first investigated. Table 1 shows that mannose treatment markedly decreased the acid soluble phosphate content by 66%. This might be caused by the decrease in phosphate level through the sequesteration of phosphate as mannose-6-phosphate (Harris *et al.*, 1986; Wilson and Lucas, 1987). Carbon fixation rate in these leaves was also decreased by 67% (Table 2). This decrease in photosynthesis was not apparently caused by the changes in photosynthetic pigment levels since there was no effect on Chl a, Chl b, total chlorophyll, and total carotenoids (Table 3). However, starch content was increased by 23% of controls with no changes in sucrose content (Table 4). The starch content was generally enhanced in response to P, depletion (Foyer *et al.*, 1982; Foyer and spencer, 1986), and has been interpreted as the disturbance of [ATP]/[ADP] ratio (Robinson and Walker, 1981) and/or the shortage of RuBP through the diversion of newly

Table 1. Changes of acid slouble phosphate content in the 15-day old primary leaves of Chinese cabbage (Co-0h) and incubated detached leaves for 12 h in growth light conditions with either DW (Co-12h) or 25 mM mannose (Ma-12h). The changes are given in percent of the control (Co- 0h). Data are means± SD (n=9)

	Co- 0h	Co-12h	Ma-12h
Pi	0.47± 0.05	0.44± 0.09	0.16± 0.03
(μg/leaf)	(100)	(93.6)	(34.1)

Table 2. Changes of CO_2 uptake rates ($\mu molCO_2 m^{-2}s^{-1}$) in the 15-day old primary leaves of Chinese cabbage (Co-0h) and incubated detached leaves for 12 h in growth light conditions with either DW (Co-12h) or 25 mM mannose (Ma-12h). The changes are given in percent of the control (Co- 0h). Data are means± SD (n=3−4)

	Co- 0h	Co-12h	Ma-12h
Photosynthesis	5.57± 0.29	5.02± 0.03	1.30± 0.11
($\mu molCO_2 m^{-2}s^{-1}$)	(100)	(90.0)	(23.0)

Table 3. Changes of Chl a, Chl b, Chl a+b, total carotenoid contents (μg/leaf) and ratio of Chl a/b (mean± SD) in the 15-day old primary leaves of Chinese cabbage (Co- 0h) and incubated of detached leaves for 12 h in growth light conditions with either DW (Co-12h) or 25 mM mannose (Ma-12h). All measurements are average of at least 3 times of triplicate in each case

	Chl a	Chl b	Chl a+b	Chl a/b	Tol. Car.
Co- 0h	17.24± 1.74	4.15± 0.33	21.39± 2.05	4.15± 0.20	2.80± 0.29
Co-12h	19.74± 1.41	4.56± 0.61	24.30± 2.02	4.32± 0.27	3.16± 0.20
Ma-12h	18.34± 1.42	4.27± 0.66	22.61± 2.11	4.29± 0.36	3.01± 0.14

Table 4. Changes of starch and sucrose contents in the 15-day old primary leaves of Chinese cabbage (Co- 0h) and incubated detached leaves for 12 h in growth light conditions with either DW (Co-12h) or 25 mM mannose (Ma-12h). The changes are given in percent of the control (Co- 0h). Data are means± SD (n=9)

	Co- 0h	Co-12h	Ma-12h
Starch (glucose equiv. mg/leaf)	32.96± 1.36 (100)	41.78± 3.54 (126.8)	49.37± 4.45 (149.8)
Sucrose (mg/leaf)	0.15± 0.01 (100)	0.11± 0.02 (75.5)	0.11± 0.02 (75.5)

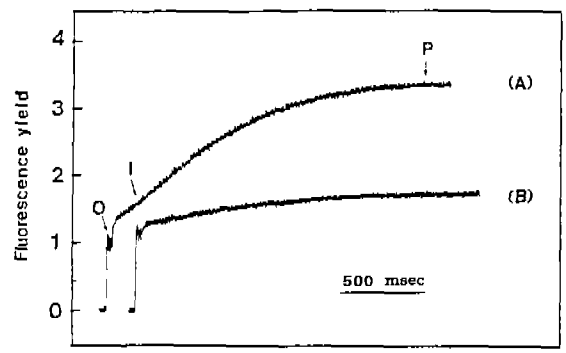

Fig. 1. Changes in chlorophyll fluorescence induction kinetics in detached Chinese cabbage leaves treated with DW (A) or 25 mM mannose for 12 h (B) in the light.

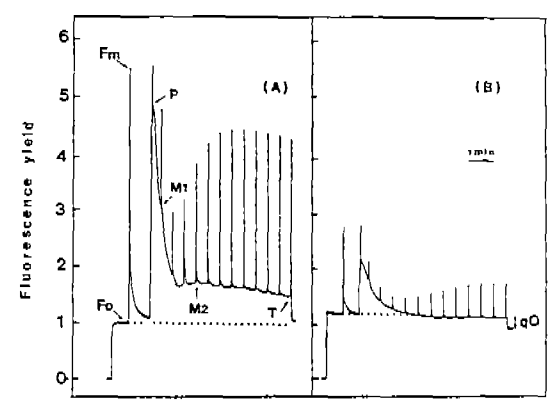

Fig. 2. Changes in the saturation pulse induction kinetics of detached Chinese cabbage leaves treated with DW (A) or 25 mM mannose for 12 h (B) in the light.

fixed CO_2 for starch synthesis (Rao *et al.*, 1989). These results clearly support the view that the rate of photosynthesis can be limited by the supply or utilization of phosphate (Sharkey, 1985; Sharkey *et al.*, 1986).

Effect on the O-I-P induction kinetics. Tuning response of PSII to the lowered P_i using room temperature chlorophyll fluorescence was investigated. Figure 1 depicts the kinetics of the two step fluorescence rise (O-I-P) at room temperature. Mannose feeding increased slightly the F_O level, which could be interpreted as the decreases in the initial energy distribution to PSII and/or efficiency of excitation trapping at PSII reaction centers (Briantais *et al.*, 1986; Krause and Weis, 1991). In contrast, there were a slight decrease in F_O-to-intermediate plateau (F_I) transient and a pronounced decrease in F_I-to peak level fluorescence (F_P) increase. Since F_I to F_P transient is a manifestation of the plastoquinone pool reduction, mannose feeding prevents the photoreduction of plastoquinone. In addition, the initial rise from F_O to F_I which reflects the photoreduction of Q_A is also affected by this treatment. It may be concluded that mannose-fed leaves showed decreases in the photoreduction of electron carriers. One possible way for interpretation above data may be the acidification of the thylakoid lumen. Indeed, it was reported that acidification of thylakoid lumen inhibited electron donation from the O_2-evolving complex to the PSII reaction center (Crofts and Horton, 1991).

Effects on the P-S-M-T induction kinetics, and photochemical and nonphotochemical quenchings. Mannose feeding also affected significantly the slow induction ki-

netics of chlorophyll fluorescence (Fig. 2). In comparision to that of control (Fig. 2A), the curve for mannose-fed leaves (Fig. 2B) exhibited: 1) a slight increase of dark level fluorescence (F_O) as shown in Fig. 1, 2) decreases of maximum (F_m) and peak level [$(F_v)_p$] fluorescence, 3) a slower quenching from F_p to steady state, 4) disappearances of M_1 shoulder and M_2 peak, 5) a decrease of F_v to below original F_O, and 6) an appearance of dark level fluorescence quenching (F_O') after actinic light off.

It has been known that the decline in the ratio of $(F_v)_m/F_m$ represents the decrease in photochemical efficiency at PSII reaction center (Krause and Weis, 1991). There are three possible explanations for the decline of $(F_v)_m/F_m$. Firstly, there may be non-radioative dissipation of excitation energy in the antenna or reaction centers (Demmig *et al.*, 1987). Secondly, there may be wasteful photochemistry resulting in Q_A being oxidized by processes that do not lead to O_2 evolution (Crofts and Horton, 1991; Schreiber *et al.*, 1989). Finally, it may be phosphorylation of PSII-associated polypeptides including the light-harvesting chlorophyll a/b complexes of PSII which could be resulted in the reduction of electron transport (Hodges *et al.*, 1987; Habashi and Baker, 1990). Although there is no direct correlations between above views, the occurrence of phosphorylation in mannose-fed leaves was indirectly noticed by the F_O quenching. This would be discussed in the later.

It has been presented that M_1 shoulder, correspondent to the burst of O_2 evolution, may be due to photoreduction of the $NADP^+$ and PGA pools before illumination, and M_2 peak, corresponding to the oscillation of O_2 evolution rate, due to an oscillation in ATP/ADP ratio (Robinson and Walker, 1981; Stitt and Schreiber, 1988). Therefore, the lacks of M transients in low phosphate leaves imply that the rates of ATP synthesis and/or consumption is too slow to induce the fluctuations of ATP/ADP. It is likely that this condition would lead to a temporary excess of assimilatory power such as ATP and NADPH, and in trun result in affecting thylakoid basic function. Changes in thylakoid function by metabolic factors can be easily detected by quenching analysis with saturation pulse method at room temperature (Schreiber *et al.*, 1986, 1989).

In Fig. 2 quenching components were analysed by applications of saturation light pulse during induction of photosynthesis by actinic light. In the control leaf (Fig. 2A), the induction kinetics of qQ initially show rapid reduction of the electron carriers (decrease of qQ to 0.3) followed by a fast reoxidation (increase of qQ to 0.9). Membrane energization (initial increase of qE to 0.5) relaxes again (decrease of qE to 0.25) through the utilization of ATP by the onset of the Calvin cycle (Schreiber *et al.*, 1986; Schreiber and Bilger, 1987). Under experimental conditions used here (Fig. 2A), the other minor components of qNP, such as state transition-related quenching (qT) and photoinhibition related quenching (qI), likely to be very limited. We can assume that qT and qI were not measured in Fig. 2 because they are significant parts of qNP at very low light conditions or at very high light intensities with a rather slow processes on average on the orders of hours (Demmig *et al.*, 1987; Havaux, 1990; Horton and Hague, 1988; Horton, 1987; Quick and Stitt, 1989), respectively. However, 12 h mannose-treated leaves (Fig. 2B) show slower reoxidation of plastoquinone (from 0.38 to 0.81) and relaxation of qE (from 0.58 to 0.35) than controls. Both decrease in F_V below the F_O level and dark level fluorecence quenching, qO (=0.22) were also occurred. These have been regarded as state transitions (Satoh and Fork, 1982; Schreiber and Bilger, 1987). It was reported that these would be induced by reduced Q_A and regulated by pH gradient occurred over a time-scale of several minutes (Bassi *et al.*, 1988; Bennet, 1991; Noctor *et al.*, 1991). Data above presented suggest that low qQ (i.e., reduction of plastoquinone) and high qE (i.e., probably build-up pH gradient in the thylakoid lumen) during early induction of photosynthesis under phosphate deficiency lead to the phosphorylation of PSII-associated polypeptides.

적 요

25 mM mannose를 광조건에서 12시간 동안 잘라낸 배추(*Brassica campestris* L.) 잎에 처리하여 광합성을 제한하였을 때 유도되는 틸라코이드 막의 기능변화를 조사하였다. Mannose 처리에 의해서 광합성 색소함량에는 변화가 없었으나 광합성 기질의 일종인 무기인산 함량과 CO_2 흡수율이 각각 66%, 67% 감소하였으며 녹말 함량은 24% 증가하였다. 빠른 엽록소 형광 유도양상에서 암수준형광 값(F_O')이 약간 증가하였으며 과도(intermediate) 수준에서 피이크 수준으로의 형광 증가가 현저하게 감소하였다. 이 결과는 무기인산 감소에 의해 광계 II 반응중심으로의 들뜬에너지 전이능과 PSII의 이차 전자수용체인 Q_B의 환원이 현저하게 저해됨을 의미한다. 포화광 조건에서 $(F_v)_m/F_m$ 비율이 감소하였는데 이는 광계 II의 들뜬에너지 포획능의 저해를 의미한다. 느린 엽록소 형광 유도양상에서 상전이를 의미하는 암수준 형광 소산(qO)이 유도되었으며, 형광은 주로 비광화학성 성분(qE+qT)에 의해 소산되었다. 이상의

결과에서 배추 잎의 무기인산이 감소함에 따라 광합성이 제한되는 경우에, 틸라코이드 막에서는 광계 II에 의한 들뜬에너지 포획능 및 반응중심으로의 전이율이 감소하며, 이 상태에서 광합성이 유도될 때에는 상전이가 수반됨을 알 수 있었다.

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