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Over-reduced states of the Mn-cluster in cucumber leaves induced by dark-chilling treatment

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Abstract

Oxygen evolution is inhibited when leaves of chilling-sensitive plants like cucumber are treated at 0 °C in the dark. The activity is restored by moderate illumination at room temperature. We examined the changes in the redox state of the Mn-cluster in cucumber leaves in the processes of dark-chilling inhibition and subsequent light-induced reactivation by means of thermoluminescence (TL). A TL B-band arising from $S_2Q_B^-$ charge recombination in PSII was observed upon single-flash illumination of untreated leaves, whereas four flashes were required to yield the B-band after dark-chilling treatment for 24 h. This three-step delay indicates that over-reduced states of the Mn-cluster such as the S_{-2} state were formed during the treatment. Fitting analysis of the flash-number dependence of the TL intensities showed that the Mn-cluster was more reduced with a longer period of the treatment and that S_{-3} was the lowest S-state detectable in the dark-chilled leaves. Measurements of the Mn content by atomic absorption spectroscopy showed that Mn atoms were gradually released from PSII during the dark-chilling treatment but re-bound to PSII by illumination at 30 °C. Thus, dark-chilling inhibition of oxygen evolution can be ascribed to the disintegration of the Mn-cluster due to its over-reduction. The observation of the S_{-3} state in the present in vivo system strongly suggests that S_{-3}, which has been observed only by addition of exogenous reductants into in vitro preparations, is indeed a redox intermediate of the Mn-cluster in the processes of its disintegration and photoactivation.

Keywords: Mn-cluster; S-state; Thermoluminescence; Oxygen-evolving complex; Cucumber (*Cucumis sativus* L.); Dark-chilling

1. Introduction

Photosynthesis is a process that produces oxygen and carbohydrate utilizing light energy. The sequential redox reactions in photosynthesis are conducted by the electron transport chain in thylakoid membranes of chloroplasts. Through electron-transfer reactions by two photosystems, electrons are ultimately extracted from water, resulting in the formation of molecular oxygen. This photosynthetic water oxidation takes place at a tetranuclear Mn-cluster located in Photosystem II (PSII) (for a review, see Ref. [1]). Oxygen-evolving reaction in the Mn-cluster is known to proceed through a cycle of five intermediates, termed S_i states ($i=0-4$) [2]. Each S-state transition, except the $S_4 \rightarrow S_0$ transition, is driven by a single flash of light via the electron transfer in PSII reaction center. The reaction is accompanied by redox changes in the Mn-cluster, and S_0 and S_4 are thought to be the most reduced and oxidized states, respectively. The S_1 state is stable in the dark and O_2 is released in the $S_4 \rightarrow S_0$ transition. It is generally accepted that the functional Mn-cluster includes high oxidation states (III, IV) of Mn (for reviews, see Refs. [3,4]).

Experiments of selective extraction of Mn ions and reassembly of the Mn-cluster have been carried out using in vitro preparations [5–7]. Treatment with Tris or millimolar concentrations of hydroxylamine releases Mn ions from PSII, and subsequent illumination in the presence of Mn^{2+} ions can induce the reassembly of the Mn-cluster. This light-induced reactivation process is known as photoactivation (for a review, see Ref. [8]). On the other hand, it has been reported that treatment with various reductants

Abbreviations: DPC, diphenylcarbazide; XANES, X-ray absorption near edge structure

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such as hydrazine [9,10], hydroxylamine [10–14], hydroquinone [12], hydrogen peroxide [15,16], and nitric oxide [17] induces the delay of flash-induced oxygen evolution, which is proposed to be due to production of over-reduced forms of the Mn-cluster such as the S_{-1}, S_{-2} and S_{-3}. These lower S-states have been postulated to be intermediates in the process of photoactivation. However, very few studies have been made on the over-reduced states of the Mn-cluster in vivo.

Cucumber is known as a chilling-sensitive plant [18], and when its leaves are treated at 0 °C in the dark, the oxygen-evolving activity markedly diminishes [19–21]. The site of damage was assumed to be the Mn-cluster in PSII because the electron-transfer activity in the presence of an artificial electron donor, diphenylcarbazide (DPC), was unchanged by the treatment [20] and oxygen evolution was fully restored by illumination at room temperature [19,21]. Thus, dark-chilling inhibition and light-induced reactivation of the oxygen evolution in cucumber leaves is an ideal system to investigate the redox properties of the Mn-cluster in the processes of disintegration and reassembly in vivo.

Thermoluminescence (TL) is a powerful tool to investigate the properties of the redox components in photosynthetic apparatus (for a review, see Refs. [22,23]). TL is an outburst of light emission from a preilluminated sample during increase of the temperature. The temperature at which TL emission occurs reflects the energetic depth of a trapped charge pair. TL has been extensively applied to the studies of PSII especially of the Mn-cluster, because the S_2 and S_3 states of the Mn-cluster exhibit specific TL bands by recombination with reduced quinone acceptors, Q_A^- and Q_B^-. The detection of TL emission provides information about the S-state distribution of the Mn-cluster under a certain condition. In addition, TL is useful to characterize the Mn-cluster in vivo since it can be measured using intact leaves. In this study, we have investigated the redox properties of the Mn-cluster in cucumber leaves in the processes of dark-chilling inhibition and subsequent photoactivation by means of TL measurement. We have observed over-reduced states of the Mn-cluster including the S_{-3} state, which is the lowest S-state ever detected in vivo.

2. Materials and methods

2.1. Plant materials

Cucumber (*Cucumis sativus* L. cv. Nanshin; Takii, Japan) plants were grown hydroponically [24] at 30 °C under the conditions of 14 h light (300 μmol photons m^{-2} s^{-1}) and 10 h darkness in a growth chamber (LPH-200-RDS; Nihonika, Japan). Fully expanded leaves were used for experiments. For dark-chilling treatment, detached leaves were wrapped in aluminum foil and then placed on ice for indicated times.

2.2. Isolation of thylakoid membranes and PSII-enriched membranes

Thylakoid membranes were isolated from cucumber leaves as described previously [24]. PSII-enriched membranes were prepared as in Ref. [20]. Thylakoid membranes from cucumber leaves were suspended (2 mg Chl/ml) in a buffer containing 0.4 M sucrose, 50 mM Mes/NaOH (pH 6.5), 10 mM NaCl, and 5 mM $MgCl_2$, and were solubilized with Triton X-100 (20 mg/mg Chl) at 4 °C for 10 min in the dark. The solubilized thylakoid membranes were centrifuged at $35,000 \times g$ for 20 min. The precipitates were resuspended in the same buffer, and then centrifuged at $5000 \times g$ for 10 min. The PSII-enriched membranes with a Chl *a*/*b* ratio of 1.9–2.1 were collected from the supernatant by centrifugation at $35,000 \times g$ for 20 min. Chlorophyll concentration was determined after extraction with 80% acetone/water according to Ref. [25].

2.3. Oxygen-evolving activity

The rate of oxygen evolution under continuous illumination (2500 μmol photons m^{-2} s^{-1}; COLD SPOT, PICL-NRX; Nippon P.I., Japan) was determined at 25 °C with a Clark-type oxygen electrode (Rank Brothers Ltd., England) in the presence of 0.2 mM 2,6-dichloro-*p*-benzoquinone as an electron acceptor. The assay mixture contained thylakoid membranes (10 μg Chl/ml), 50 mM Mes/NaOH (pH 6.5), 0.4 M sucrose, 10 mM NaCl and 5 mM $MgCl_2$.

2.4. Thermoluminescence measurement

TL glow curves were measured with a home-built apparatus [26]. Dark-adapted cucumber leaves were excited at 10 °C by saturating xenon flashes (SL-230S; Sugawara, Japan) with a repetition rate of 1 Hz, and then rapidly frozen to about −180 °C by spurting cold N_2 gas. The frozen samples were then heated at a constant rate (40 °C/min) and TL emission was detected with a photomultiplier (Hamamatsu, R943-02).

2.5. Determination of Mn content

PSII-enriched membranes were washed twice with a buffer containing 50 mM Mes/NaOH (pH 6.5), 0.4 M sucrose, 15 mM NaCl and 2 mM EDTA and then finally diluted with MilliQ (Autopore WR 600A; Yamato, Japan) water. The Mn content of the PSII-enriched membranes was determined with an atomic absorption spectrophotometer (AA-6800, Shimadzu, Japan) equipped with a graphite furnace atomizer (GFA-EX7, Shimadzu).

2.6. Calculation of S-state distribution

S-state distributions of the Mn-cluster in cucumber leaves were calculated from the flash-number dependence

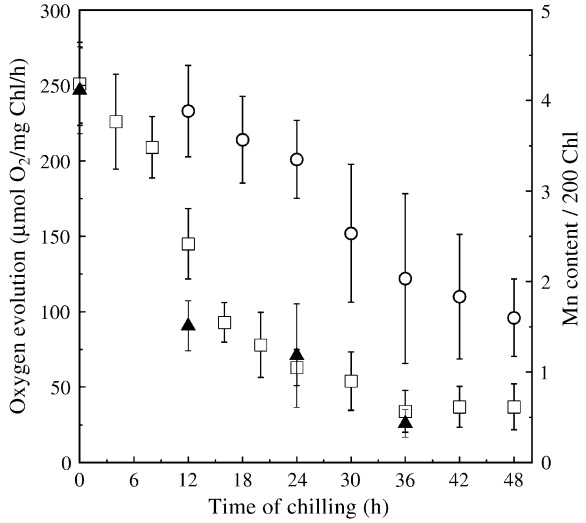

Fig. 1. Effect of dark-chilling treatment on the oxygen-evolving activity and Mn content of cucumber leaves. Cucumber leaves were incubated at 0 °C in the dark and then illuminated (80 μmol photons m^{-2} s^{-1}) at 30 °C for 30 min. Oxygen evolution was measured using thylakoid membranes isolated from dark-chilled leaves (open squares) and illuminated leaves after dark-chilling treatment (open circles). Closed triangles show Mn content in PSII-enriched membranes isolated from cucumber leaves after dark-chilling treatment. The data represent the averages of three to six experiments.

of TL intensities by least-squares fitting following the method of Messinger et al. [27]. The probabilities of misses and double hits were assumed to be the same for all S-state transitions. These two parameters and the luminescence yields of the $S_3Q_B^-$ and $S_2Q_B^-$ charge recombination were determined in untreated cucumber leaves. S-state distributions including the S_{-1}, S_{-2} and S_{-3} states in dark-chilled leaves were then calculated with these parameters fixed. Before measurements, untreated leaves were preilluminated with white light of 10 W/m^2 for 30 s, followed by a 5-min dark-adaptation period at room temperature.

3. Results

Fig. 1 shows the oxygen-evolving activity of thylakoid membranes isolated from the cucumber leaves that were treated at 0 °C in the dark for 0–48 h. The activity decreased with an increase in the dark-chilling treatment (open squares) as reported previously [20]. When these dark-chilled leaves were illuminated with low-intensity light (80 μmol photons m^{-2} s^{-1}) at a growth temperature (30 °C) for 30 min, oxygen evolution was significantly restored (open circles). The extent of reactivation decreased in the leaves dark-

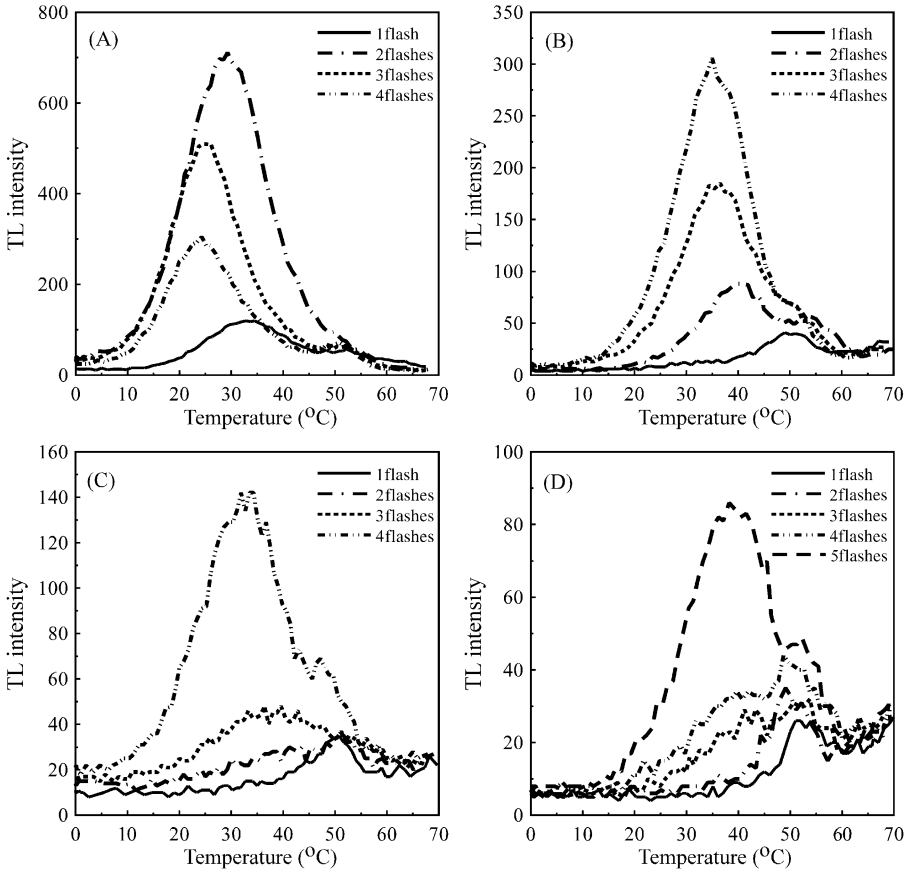

Fig. 2. TL grow curves of cucumber leaves dark-chilled for 0 (A), 12 (B), 24 (C), and 36 (D) h. The leaves were excited with a series of saturating flash (1 Hz) at 10 °C before freezing.

chilled for a longer period, e.g. 48 h, suggesting that a part of PSII was irreversibly inhibited by longer treatment. This irreversible inhibition might be caused by damage to the acceptor side of PSII or death of cells and tissues. When cucumber leaves were incubated at room temperature in the dark for 24 h, oxygen evolution retained more than 80% of the activity of untreated leaves. This indicates that not only darkness but also low temperature is a prerequisite for the inhibition of oxygen evolution. Closed triangles show the Mn content in the PSII-enriched membranes isolated from cucumber leaves in the course of dark-chilling treatment. The Mn content and the oxygen-evolving activity were well correlated with each other, suggesting that the direct cause for the decrease in oxygen evolution by dark-chilling treatment is the release of Mn from PSII complexes. When the dark-chilled leaves were illuminated with photon flux density at more than 150 μmol photons m^{-2} s^{-1}, oxygen evolution did not recover completely. When the oxygen-evolving capacity is eliminated by Tris treatment, PSII reaction center became susceptible to photoinhibition compared to intact samples [28]. Dark-chilled leaves might also be vulnerable to light damage due to the release of Mn.

Fig. 2 shows TL glow curves of untreated leaves (A) and of the leaves dark-chilled for 12 (B), 24 (C), and 36 (D) h.

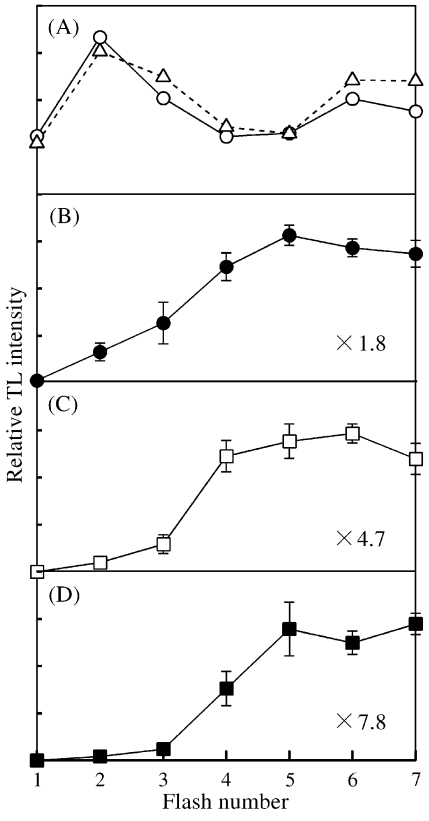

Fig. 3. Flash-number dependence of TL B-band intensities of cucumber leaves dark-chilled for 0 (A, open circles), 12 (B), 24 (C), and 36 (D) h. Data of at least four independent experiments were averaged. Open triangles show flash-number dependence of TL B-band intensities for the leaves that were incubated at room temperature in the dark for 24 h.

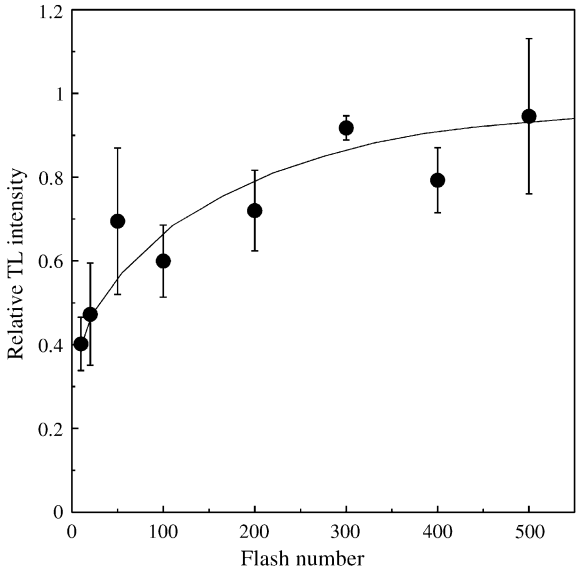

Fig. 4. Relative TL intensities of cucumber leaves dark-chilled for 24 h after excitation with a series of flashes (1 Hz). Data of at least three independent experiments were averaged.

TL B-bands arising from S_2/Q_B^- or S_3/Q_B^- recombination showed a peak at 20–40 °C at every number of flashes in the untreated leaves. In contrast to untreated leaves, the TL B-band was not detected upon the first flash in 12-h-treated leaves (Fig. 2B) (note that the band at about 50 °C is probably the C-band arising from the Y_D^+ radical [29] and commonly observed in all the samples). A B-band-like signal was observed at about 40 °C after two flashes. These observations indicate that the Mn-cluster is not in the S_1

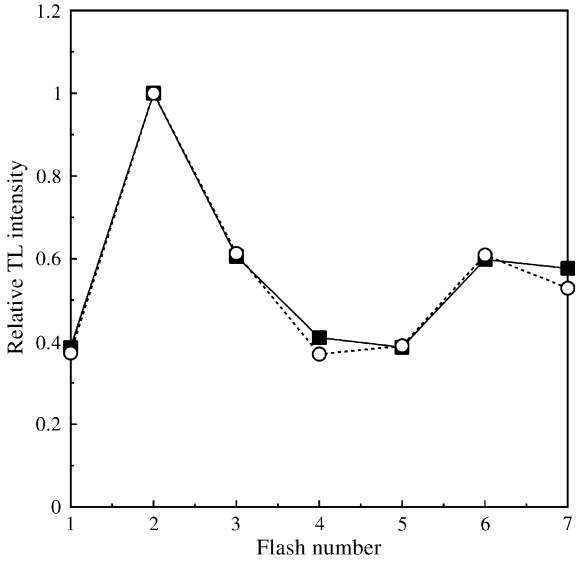

Fig. 5. Recovery of flash-number dependence of TL B-band intensities. Cucumber leaves were incubated at 0 °C in the dark for 12 h and then illuminated (80 μmol photons m^{-2} s^{-1}) at room temperature for 30 min (closed squares). Data of three independent experiments were averaged. Open circles show flash-number dependence of TL B-band intensities for the untreated leaves.

state any more but in the S_0 or even lower S-states. More numbers of flashes were required to induce sufficient B-band in the 24- and 36-h-treated leaves (Fig. 2C,D). The first strong B-band signal was observed at the fourth and fifth flash in the leaves dark-chilled for 24 and 36 h, respectively. The TL intensity plotted against flash number showed a typical pattern of period-four oscillation with maxima at the second and sixth flash in the untreated leaves (Fig. 3A, open circle). Flash-number dependence of TL intensities did not show an obvious oscillation pattern in the dark-chilled leaves (Fig. 3B–D), indicating that the Mn-cluster does not exist in a single redox state but rather in a mixture of different states. Open triangles in Fig. 3A show flash-number dependence of TL B-band intensities for the leaves that were incubated at room temperature in the dark for 24 h. Oscillation pattern of these leaves was almost the same as untreated leaves, indicating that the dark incubation of leaves at room temperature did not cause the modification of the Mn-cluster.

In addition to the increase of flash number to give the first strong TL signal, absolute TL intensity was significantly altered after dark-chilling treatment. As shown in Fig. 2, the maximum TL intensity in relative units decreased from about 700 (at second flash) in untreated leaves to about 300 (fourth flash), 140 (fourth flash), and 90 (fifth flash) after dark-chilling treatment for 12, 24 and 36 h, respectively. The TL intensities were, however, gradually recovered by illumination with a larger number of flashes. Fig. 4 shows the B-band intensity of the 24-h-treated leaves upon excitation with consecutive flashes (1 Hz) at 10 °C. The TL intensity increased with more flashes, and the intensity upon

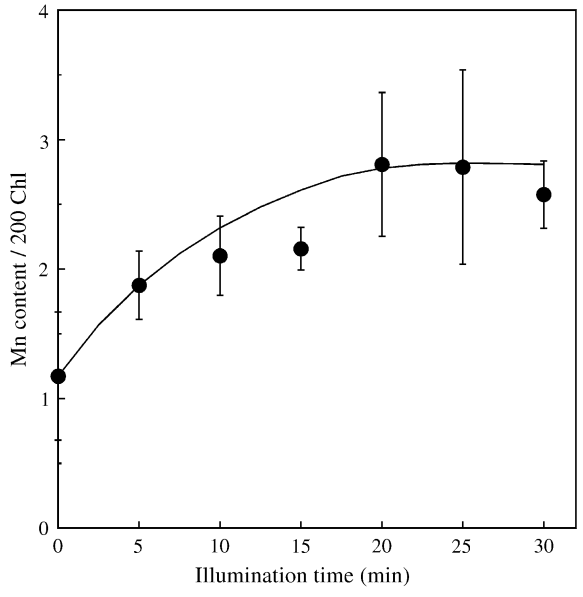

Fig. 6. Mn content in PSII-enriched membranes of dark-chilled cucumber leaves after continuous illumination. Cucumber leaves were dark-chilled for 24 h and then illuminated at 80 μmol photons m^{-2} s^{-1} at 30 °C for indicated times. Data of at least three independent experiments were averaged.

500 flashes reached about 80% of that of the 24-h-treated leaves reactivated with continuous illumination for 30 min. When the leaves dark-chilled for 12 h were illuminated at room temperature for 30 min, oscillation of the B-band intensity was also restored, i.e. it exhibited a period-four oscillation (Fig. 5). This is consistent with the result that oxygen evolution recovered after illumination for 30 min (Fig. 1). After the dark-chilling treatment for 24 h, however, the leaves illuminated for 30 min did not show typical oscillation pattern although the B-band signal was observed at the first flash (data not shown). This might be caused by modification of the Q_B site with longer dark-chilling treatment.

The Mn content was also measured during the reactivation process of the leaves dark-chilled for 24 h (Fig. 6). The Mn content in PSII gradually increased and reached saturation after illumination for about 20 min, indicating that the Mn-cluster was reassembled in PSII.

4. Discussion

When cucumber leaves were subjected to dark-chilling treatment, the number of flashes necessary to induce significant TL B-bands increased along with the decrease of the TL intensity. Because this observation was concomitant with the decrease in oxygen-evolving activity and the Mn content in PSII (Fig. 1), the delay of flash-number dependence can be ascribed to over-reduced forms of the Mn-cluster produced as intermediates during the disintegration process of the cluster. For instance, about 70% of Mn was released from PSII in the leaves dark-chilled for 24 h (Fig. 1), and the remaining Mn-cluster was distributed in the over-reduced states, presumably in the S_{-2} state, since four flashes are required for the observation of the first intense TL band (Fig. 2).

The S-state distribution in the remaining Mn-cluster in dark-chilled leaves was estimated by least-squares fitting of the flash-number dependence of TL intensities (Fig. 3). The parameters of miss and double-hit probabilities and the intensity ratio of luminescence from $S_3Q_B^-$ and $S_2Q_B^-$ recombination were calculated using the oscillation pattern of untreated leaves (Fig. 3A) assuming the S-state populations of 75% S_1 and 25% S_0 and 1:1 distribution of Q_B and Q_B^-. The best fit was obtained with 15.9% misses and 8.6% double hits and with the luminescence ratio of 4.0. The S-state distributions including the over-reduced states of S_{-1}, S_{-2} and S_{-3} in dark-chilled leaves were calculated with these parameters fixed. The obtained result in Table 1 shows that the population of the distribution shifted to the lower S-states with longer dark-chilling treatment. After the treatment for 12 h, the S_0 and S_1 populations markedly decreased and more than 80% of the remaining Mn-cluster was in the over-reduced states (S_{-1}, S_{-2} and S_{-3}). The major population of the Mn-cluster was in the S_{-2} state after 24 h dark-chilling treatment. In 36-h-treated leaves,

Table 1
Estimated initial S-state populations of cucumber leaves under dark-chilling conditions

Chilling time (h)	S-state population (%)[a]					Mn content in PSII (%)
	S_1	S_0	S_{-1}	S_{-2}	S_{-3}	
0[b]	75.0	25.0	0.0	0.0	0.0	100.0
12	5.7 (2.1)[c]	6.2 (2.3)	26.6 (9.8)	36.6 (13.4)	24.9 (9.2)	36.7
24	0.1	0.0	28.9 (8.3)	35.9 (10.3)	35.1 (10.1)	28.7
36	0.0	0.0	11.7 (1.2)	26.8 (2.8)	61.5 (6.5)	10.6

[a] S-state populations were calculated by least-squares fitting of the flash-number dependence of TL B-band intensities (Fig. 3). Miss and double-hit probabilities for all S-state transitions were fixed to 15.9% and 8.6%, respectively, which were determined for untreated leaves.
[b] Initial S-state populations of untreated leaves were assumed to be 25% S_0 and 75% S_1.
[c] Numbers in parentheses are the S-state populations calculated by taking account of the Mn content in PSII.

more than half of the Mn-cluster was in the S_{-3} states. In spinach chloroplast, the intensity ratio of luminescence from $S_3Q_B^-$ and $S_2Q_B^-$ recombination is estimated to be 1.7 [30]. The ratio determined in the present study (4.0) is much higher than this value, and this may be due to the specific characters of PSII in cucumber. To check whether the difference in the intensity ratio of luminescence from $S_3Q_B^-$ and $S_2Q_B^-$ recombination influences the calculated S-state distribution, we also calculated the S-state distribution using the value of 1.7 as the intensity ratio. The best fit was obtained with 19.4% misses and 3.3% double hits. S-state distribution thus calculated showed similar pattern as Table 1. Difference of S-state distribution between two fittings was less than 10%, so that the difference in the TL intensity ratio does not influence the conclusion on the S-state distribution obtained here.

With the data of 24- and 36-h-treated leaves, it might be difficult to determine whether S_{-3} state actually exists or not because the leaves illuminated for 30 min did not show period-four oscillation. Nevertheless, the fitting clearly shows that S_{-3} state can be detected in 12-h-treated leaves that showed normal oscillation patterns after photoactivation (Fig. 5). It should be noted that fraction of S_{-4} and S_{-5} were negligible in the fitting analysis, although measurements with eight to nine flashes may be necessary for precise analysis for these states. In addition, no further delay in the flash number for the appearance of TL B-band was observed in the leaves dark-chilled for more than 36 h (data not shown). Thus, the S_{-3} state is presumably the lowest S-state detectable in cucumber leaves under dark-chilling conditions.

There have been a few studies that reported the presence of over-reduced states of the Mn-cluster in vivo. The S_{-1} and S_{-2} states were observed in the mutant either lacking the PsbO protein [31,32] or having short deletions in the lumenal E loop of the CP47 protein [33,34]. To our knowledge, however, the present study is the first case in which the S_{-3} state was observed in vivo. In in vitro studies, the S_{-3} state has been obtained by the treatment of exogenous reductants. Messinger et al. [9] reported the relatively stable S_{-3} state in hydrazine-treated thylakoids, while hydroxylamine and hydroquinone appeared to form unstable S_{-3} in PSII preparations [12,14]. Messinger et al. [10] also reported the presence of the S_{-4} and S_{-5} states in hydrazine-treated thylakoids of *Synechococcus elongatus*, although these intermediates were relatively unstable.

The possibility was previously argued that the S_{-3} state produced in vitro may actually reflect the S_{-1} (or S_{-2}) state with a reductant bound at the catalytic site [9]. Our result that S_{-3} state was detected in cucumber leaves under dark-chilling condition strongly suggests that the S_{-3} state found in vitro is not due to a bound reductant but a genuine redox intermediate of the Mn-cluster, because our experiments were carried out under physiological condition without any reductant. The possibility, however, is not strictly excluded that an endogenous reductant is bound to the Mn-cluster in cucumber leaves and is responsible for the flash-number delay.

TL intensities of 24-h-treated leaves exhibited a sudden increase upon the fourth flash (Fig. 3C), which provides about 30% of the saturation level, followed by slow increase of the TL intensity by a larger number of flashes (Fig. 4). This slow TL increase was correlated with the recovery of oxygen evolution (Fig. 1) and the increase in the Mn content in PSII (Fig. 6). These observations indicate that the over-reduced Mn-cluster that retains the basic structure of a tetranuclear cluster can be activated by several flashes with high efficiency, whereas released Mn^{2+} ions are reassembled by a photoactivation process with much lower efficiency. In the process of photoactivation, the over-reduced states of the Mn-cluster are thought to be transient intermediates [8,35]. The first stable intermediate in photoactivation is formed by two-flash oxidation of Mn^{2+} ions with an interval of a dark step, leading to the view that this stable intermediate has an oxidation state of $(II)_2(III)_2$ (including a $(III)_2$ dimer and two free Mn^{2+}) [5–8,36,37]. In the current study, it is considered that the S_{-3} state is the lowest stable S-state. Thus, assuming single oxidation of Mn upon each flash, the oxidation state of the S_1 state is presumed to be $(III)_2(IV)_2$, which is consistent with the previous conclusion of X-ray absorption near edge structure (XANES) studies [12,38,39].

The question still remains as to why the Mn-cluster is reduced under dark-chilling conditions. It was reported that tonoplast H^+-ATPase is uncoupled at chilling temperature in the dark [40]. The change in cellular pH due to this

uncoupling could inactivate the Mn-cluster. Dark-chilling treatment of cucumber leaves was also reported to cause dissociation of the PsbP and PsbQ proteins from PSII [20]. The presence of these two proteins was shown to protect the Mn-cluster from the effects of reductants, such as hydroquinone [41]. Since these proteins dissociate from PSII at acidic pH [42], their release by the change in cellular pH might promote reduction of the Mn-cluster. Thus, the Mn-cluster might be disintegrated by the attack of bulk endogenous reductants under dark-chilling condition. Ascorbate is known to serve as a reductant in the violaxanthin deepoxidase reaction in the thylakoid lumen [43,44]. The Mn-cluster might be reduced by endogenous reductants such as ascorbate as a consequence of cellular acidification. Further investigation is required to answer this question.

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References

[1] R.J. Debus, The manganese and calcium ions of photosynthetic oxygen evolution, Biochim. Biophys. Acta 1102 (1992) 269–352.
[2] B. Kok, B. Forbush, M. McGloin, Cooperation of charges in photosynthetic O_2 evolution: 1. A linear four-step mechanism, Photochem. Photobiol. 11 (1970) 457–475.
[3] R.D. Britt, Oxygen evolution, in: D.R. Ort, C.F. Yocum (Eds.), Oxygenic Photosynthesis: The Light Reactions, Advances in Photosynthesis, vol. 4, Kluwer Academic Publishers, Dordrecht, Netherlands, 1996, pp. 137–164.
[4] V.K. Yachandra, K. Sauer, M.P. Klein, Manganese cluster in photosynthesis: where plants oxidize water to dioxygen, Chem. Rev. 96 (1996) 2927–2950.
[5] N. Tamura, G.M. Cheniae, Photoactivation of the water-oxidizing complex in Photosystem II membranes depleted of Mn and extrinsic proteins: 1. Biochemical and kinetic characterization, Biochim. Biophys. Acta 890 (1987) 179–194.
[6] T. Ono, Y. Inoue, Reductant-sensitive intermediates involved in multiquantum process of photoactivation of latent oxygen-evolving system, Plant Cell Physiol. 28 (1987) 1293–1300.
[7] L. Zaltsman, G.M. Ananyev, E. Bruntrager, G.C. Dismukes, Quantitative kinetic model for photoassembly of the photosynthetic water oxidase from its inorganic constituents: requirements for manganese and calcium in the kinetically resolved steps, Biochemistry 36 (1997) 8914–8922.
[8] T. Ono, Metallo-radical hypothesis for photoassembly of $(Mn)_4$-cluster of photosynthetic oxygen evolving complex, Biochim. Biophys. Acta 1503 (2001) 40–51.
[9] J. Messinger, G. Seaton, T. Wydrzynski, U. Wacker, G. Renger, S_{-3} state of the water oxidase in Photosystem II, Biochemistry 36 (1997) 6862–6873.
[10] J. Messinger, J.H. Robblee, U. Bergmann, C. Fernandez, P. Glatzel, S. Isgandarova, B. Hanssum, G. Renger, S.P. Crammer, V.K. Yachandra, Manganese oxidation states in Photosystem II, Proceedings of the 12th International Congress on Photosynthesis, CSIRO Publishing, Collingwood, Australia, 2001, pp. S10–S19.
[11] B. Bouges, Action of low concentrations of hydroxylamine on oxygen evolved by Chlorella and spinach chloroplasts, Biochim. Biophys. Acta 234 (1971) 103–112.
[12] P.J. Riggs-Gelasco, R. Mei, C.F. Yocum, J.E. Penner-Hahn, Reduced derivatives of the Mn cluster in the oxygen-evolving complex of Photosystem II: an EXAFS study, J. Am. Chem. Soc. 118 (1996) 2387–2399.
[13] H. Kretschmann, H.T. Witt, Chemical reduction of the water splitting enzyme system of photosynthesis and its light-induced reoxidation characterized by optical and mass spectrometric measurements: a basis for the estimation of the states of the redox active manganese and of water in the quaternary oxygen-evolving S-state cycle, Biochim. Biophys. Acta 1144 (1993) 331–345.
[14] W.F. Beck, G.W. Brudvig, Reaction of hydroxylamine with the electron–donor side of Photosystem II, Biochemistry 26 (1987) 8285–8295.
[15] B.R. Velthuys, B. Kok, Photosynthetic oxygen evolution from hydrogen peroxide [spinach chloroplasts], Biochim. Biophys. Acta 502 (1978) 211–221.
[16] J. Mano, M.-A. Takahashi, K. Asada, Oxygen evolution from hydrogen peroxide in Photosystem II: flash-induced catalytic activity of water-oxidizing Photosystem II membranes, Biochemistry 26 (1987) 2495–2501.
[17] G. Schansker, C. Goussias, V. Petrouleas, A.W. Rutherford, Reduction of the Mn cluster of the water-oxidizing enzyme by nitric oxide: formation of an S_{-2} state, Biochemistry 41 (2002) 3057–3064.
[18] K. Sonoike, Various aspects of photosynthesis under light/chilling stress: ''photoinhibition at chilling temperatures'' versus ''chilling damage in the light'', J. Plant Res. 111 (1998) 121–129.
[19] Z. Kaniuga, B. Sochanowicz, J. Zabek, K. Krzystyniak, Photosynthetic apparatus in chilling-sensitive plants: 1. Reactivation of Hill reaction activity inhibited on the cold and dark storage, Planta 140 (1978) 121–128.
[20] J.R. Shen, I. Terashima, S. Katoh, Cause for dark, chilling-induced inactivation of photosynthetic oxygen-evolving system in cucumber leaves, Plant Physiol. 93 (1990) 1354–1357.
[21] I. Terashima, L.K. Huang, C.B. Osmond, Effects of leaf chilling on thylakoid functions, measured at room temperature, in *Cucumis sativus* L. and *Oryza sativa* L., Plant Cell Physiol. 30 (1989) 841–850.
[22] I. Vass, Govindjee, Thermoluminescence from the photosynthetic apparatus, Photosynth. Res. 48 (1996) 117–126.
[23] Y. Inoue, Biophysical techniques in photosynthesis, in: J. Amesz, J. Hoff (Eds.), Photosynthetic Thermoluminescence as a Simple Probe of Photosystem II Electron Transport, Advances in Photosynthesis vol. 3, Kluwer Academic Publishers, Dordrecht, Netherlands, 1996, pp. 93–107.
[24] I. Terashima, Y. Kashino, S. Katoh, Exposure of leaves of *Cucumis sativus* L. to low temperatures in the light causes uncoupling of thylakoids: I. Studies with isolated thylakoids, Plant Cell Physiol. 32 (1991) 1267–1274.
[25] R.J. Porra, W.A. Thompson, P.E. Kriedemann, Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophyll *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy, Biochim. Biophys. Acta 975 (1989) 384–394.
[26] T. Noguchi, M. Katoh, Y. Inoue, A new system for detection of thermoluminescence and delayed luminescence from photosynthetic apparatus with precise temperature control, Spectroscopy 16 (2002) 89–94.
[27] J. Messinger, U. Wacker, G. Renger, Unusual low reactivity of the water oxidase in redox state S_3 toward exogenous reductants—analysis of the NH_2OH-induced and NH_2NH_2-induced modification of

flash-induced oxygen evolution in isolated spinach thylakoids, Biochemistry 30 (1991) 7852–7862.
[28] H.J. Eckert, B. Geiken, J. Bernarding, A. Napiwotzki, H.J. Eichler, G. Renger, 2 sites of the electron-transfer in oxygen evolving and Tris-treated PS II membrane fragments from spinach, Photosynth. Res. 27 (1991) 97–108.
[29] G.N. Johnson, A. Boussac, A.W. Rutherford, The origin of 40–50 °C thermoluminescence bands in Photosystem II, Biochim. Biophys. Acta 1184 (1994) 85–92.
[30] A.W. Rutherford, G. Renger, H. Koike, Y. Inoue, Thermoluminescence as a probe of Photosystem II, the redox and protonation states of the secondary acceptor quinone and the O_2-evolving enzyme, Biochim. Biophys. Acta 767 (1984) 548–556.
[31] R.L. Burnap, J.R. Shen, P.A. Jursinic, Y. Inoue, L.A. Sherman, Oxygen yield and thermoluminescence characteristics of a cyanobacterium lacking the manganese-stabilizing protein of Photosystem II, Biochemistry 31 (1992) 7404–7410.
[32] D.H. Engels, A. Lott, G.H. Schmid, E.K. Pistorius, Inactivation of the water-oxidizing enzyme in manganese stabilizing protein-free mutant cells of the cyanobacteria *Synechococcus* PCC7942 and *Synechocystis* PCC6803 during dark incubation and conditions leading to photoactivation, Photosynth. Res. 42 (1994) 227–244.
[33] H.M. Gleiter, E. Haag, J.R. Shen, J.J. Eaton-Rye, Y. Inoue, W.F. Vermaas, G. Renger, Functional characterization of mutant strains of the cyanobacterium *Synechocystis* sp. PCC 6803 lacking short domains within the large, lumen-exposed loop of the chlorophyll protein CP47 in Photosystem II, Biochemistry 33 (1994) 12063–12071.
[34] H.M. Gleiter, E. Haag, J.R. Shen, J.J. Eaton-Rye, A.G. Seeliger, Y. Inoue, W.F. Vermaas, G. Renger, Involvement of the CP47 protein in stabilization and photoactivation of a functional water-oxidizing complex in the cyanobacterium *Synechocystis* sp. PCC 6803, Biochemistry 34 (1995) 6847–6856.
[35] G. Renger, Photosynthetic water oxidation to molecular oxygen: apparatus and mechanism, Biochim. Biophys. Acta 1503 (2001) 210–228.
[36] G.M. Cheniae, I.F. Martin, Photoactivation of the manganese catalyst of O_2 evolution: 1. Biochemical and kinetic aspects, Biochim. Biophys. Acta 253 (1971) 167–181.
[37] M. Miyao-Tokutomi, Y. Inoue, Improvement by benzoquinones of the quantum yield of photoactivation of photosynthetic oxygen evolution: direct evidence for the two-quantum mechanism, Biochemistry 31 (1992) 526–532.
[38] V.K. Yachandra, V.J. DeRose, M.J. Latimer, I. Mukerji, K. Sauer, M.P. Klein, Where plants make oxygen: a structural model for the photosynthetic oxygen-evolving manganese cluster, Science 260 (1993) 675–679.
[39] M. Kusunoki, T. Ono, T. Noguchi, Y. Inoue, H. Oyanagi, Manganese K-edge X-ray absorption spectra of the cyclic S-states in the photosynthetic oxygen-evolving system, Photosynth. Res. 38 (1993) 331–339.
[40] S. Yoshida, C. Matsuura-Endo, Comparison of temperature dependency of tonoplast proton translocation between plants sensitive and insensitive to chilling, Plant Physiol. 95 (1991) 504–508.
[41] C.M. Waggoner, C.F. Yocum, Selective depletion of water-soluble polypeptides associated with Photosystem II, in: J. Biggins (Ed.), Progress in Photosynthesis Research, vol. 1, Martinus Nijhoff, Dordrecht, 1987, pp. 685–688.
[42] J.R. Shen, Y. Inoue, Low pH-Induced dissociation of three extrinsic proteins from O_2-evolving Photosystem-II, Plant Cell Physiol. 32 (1991) 453–457.
[43] N. Sminoff, Ascorbate biosynthesis and function in photoprotection, Philos. Trans. R. Soc. Lond., B 355 (2000) 1455–1464.
[44] K.K. Niyogi, Photoprotection revisited: genetic and molecular approaches, Annu. Rev. Plant Physiol. Plant Mol. Biol. 50 (1999) 1121–1134.