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Functional Plant Biology

Supplementary Material

A new method for separate evaluation of PSII with inactive oxygen evolving complex and active D1 by the pulse-amplitude modulated chlorophyll fluorometry

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Fig. S1. Spectra of the light sources. LEDs used for photoinhibitory treatments (*a*) and sunlight on a clear day (*b*). These spectra were measured with a handheld portable Light Analyzer LA-105 (Nippon Medical & Chemical Instruments Co., Ltd., Japan).

Fig. S2. Effects of 1% DMSO and ethanol (EtOH) on F_v/F_m. F_v/F_m in the leaves infiltrated with water, 1% EtOH, 1% DMSO, 100 μM DCMU in 1% EtOH or 100 μM in 1%DMSO were measured after the dark-chilling treatment of the leaves for 48 h. For a comparison, 1% ethanol solution was also used. F_v/F_m in the leaves infiltrated with the aqueous solution of 1% DMSO or ethanol did not differ from that in water-infiltrated leaves. Thus, in this study, we used 1% DMSO solution to dissolve DCMU. Each bar represents the mean ± SD ($n \geq 3$). Differences from the H_2O control were examined by the Dunnett-test. For comparison of DCMU samples, Student's *t* test was conducted: n.s., $P > 0.05$.

Fig. S3. Effects of the short-term and prolonged dark treatment at 23˚C after the dark-chilling treatment for 48 h on F_v/F_m. (*a*), Effect of short-term dark treatments at 23˚C after the dark-chilling treatment for 48 h on F_v/F_m. The leaves dark-chilled for 48 h were kept in the dark at 23˚C for 0, 1 and 2 h and then F_v/F_m were measured without infiltration of chemicals. F_v/F_m at 0 min was determined immediately after the chilling treatment. (*b*) F_v/F_m were measured after the infiltration of DCMU or water after the dark treatment at 23˚C for 48 h following the dark-chilling treatment for 48 h. Each bar represents the mean ± SD ($n \geq 3$). (*a*) Differences from the 0 h control were examined by the Dunnett-test: n.s., $P > 0.05$. (*b*) The difference between H_2O and DCMU was examined by the Student's *t*-test: **, $P < 0.01$. F_v/F_m in the leaves after the dark-chilling treatment did not change with the subsequent dark treatment at 23˚C for up to 48 h. The DCMU infiltration to the leaves increased F_v/F_m to 0.64 ±

0.037 from the level of water-infiltrated leaves (0.48 ± 0.060) but did not reach the levels before the chilling treatment (0.79, data not shown).

Fig. S4. Changes in the chlorophyll fluorescence transient in cucumber leaves before and after the dark-chilling treatment for 48 h. Changes in chlorophyll fluorescence level with time (the Kautsky transient) in water-infiltrated leaves (*a* and *e*) and DCMU-infiltrated leaf (*b* – *d*, *f* – *h*) of the cucumber plants before (*a* – *d*) and after (*e* – *h*) the dark-chilling treatment for 48 h. DCMU at the concentrations of 0 (1% DMSO) (*a* and *e*), 50 (*b* and *f*), 100 (*c* and *g*) and 200 μM (*d* and *h*) were vacuum-infiltrated to the leaf segments in a 30 mL syringe. Arrows indicate the onsets of the measuring light (ML) and the actinic light at 300 μmol m^{-2} s^{-1} (AL). Upon the actinic light application, chlorophyll fluorescence levels in water- and 50 μM DCMU-infiltrated, unstressed leaves promptly increased and then decreased gradually, exhibiting typical Kautsky transients. In the 100 and 200 μM DCMU-infiltrated leaves, chlorophyll fluorescence attained the maximum level and remained at this level, indicating the complete inhibition of the photosynthetic electron transport in PSII. In contrast, after the dark-chilling treatment, water-infiltrated leaf showed only a little increase upon the actinic light application. DCMU-infiltrated leaves showed the transient increases to the constant level higher than the maximum level of water-infiltrated leaf, indicating that DCMU allowed Q_A in PSII with inactive OEC to be reduced. From these results, we decided to apply DCMU at 100 μM in this study.

Fig. S5. Fluorescence kinetics induced by the 300 ms-SP after the dark-chilling treatment for 48 h (*a*) and red light at 2000 μmol m^{-2} s^{-1} for 30 min (*b*) in the cucumber leaf. Red light treatment in (*b*) was carried out at 23°C in a leaf treated with 1 mM lincomycin. The measurement procedure was the same as for Fig. 1. Traces are shown on the normal scale. The SP for 300 ms was long enough to attain the maximal fluorescence level.

Fig. S6. Effects of the dark-chilling treatment of cucumber leaves on F_o (*a*) and F_v/F_m (*b*) measured after infiltration with 100 μM DCMU. F_v/F_m in the water-infiltrated leaves decreased with the dark-chilling treatment with time, while F_o was almost constant. F_v/F_m in DCMU-infiltrated leaves was decreased by the dark-chilling treatment for 6 h but stayed at a steady level afterwards. Thus, the difference in F_v/F_m between DCMU-infiltrated and water-infiltrated leaves became greater gradually after 6 h. Each data point represents the mean ± SD ($n \geq 4$).

Fig. S7. Excitation spectra for the chlorophyll fluorescence at 690 nm of the cucumber leaves. The leaf segment was obliquely placed in a 1 cm optical cell so that the excitation light was illuminated at the incident angle of 45° and fluorescence emitted was collected at 45° of the leaf surface. Excitation spectra from 300 to 500 nm (slit width 5 nm) for the fluorescence emission at 690 nm (slit width 10 nm) were measured with a fluorescence spectrophotometer (F-2700, Hitachi, Japan). Chlorophyll fluorescence was excited from the adaxial- (grey line) or abaxial- (dashed line) side and detected from

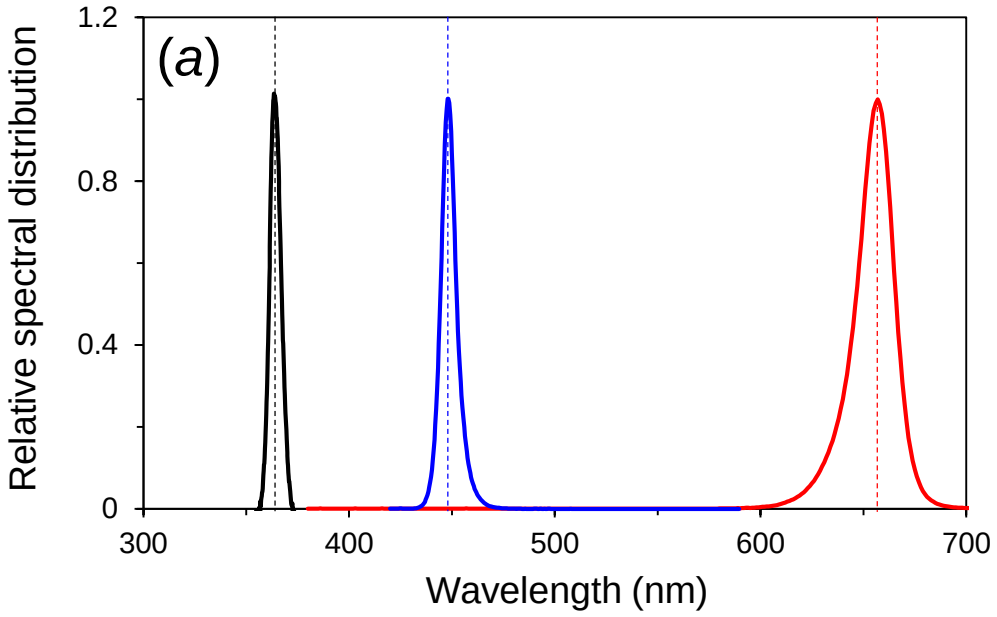
the adaxial- (grey line) or abaxial- (dashed line) side, respectively. The average of three leaves are shown with the mean ± SD. When UV-A was illuminated from the leaf adaxial side, red chlorophyll fluorescence was hardly visible to the naked eye. In contrast, when UV-A was illuminated from the leaf abaxial side, the fluorescence from both sides could be clearly seen (data not shown). Transmittance of UV light in the adaxial epidermis peel was also significantly lower than that in the abaxial one, especially in a range of 300 to 380 nm (data not shown). Chlorophyll fluorescence intensity from the leaf adaxial surface was markedly lower for the excitation wavelengths ranging from 300 to 360 nm than that from the abaxial surface. These results indicate that the adaxial epidermis of the cucumber leaf had UV-absorbing compounds and they protected the chloroplasts in the mesophyll cells from the incident UV-A. This would explain why F_v/F_m in the cucumber leaves was not decreased by exposure of the adaxial side to UV-A (Fig. 5).

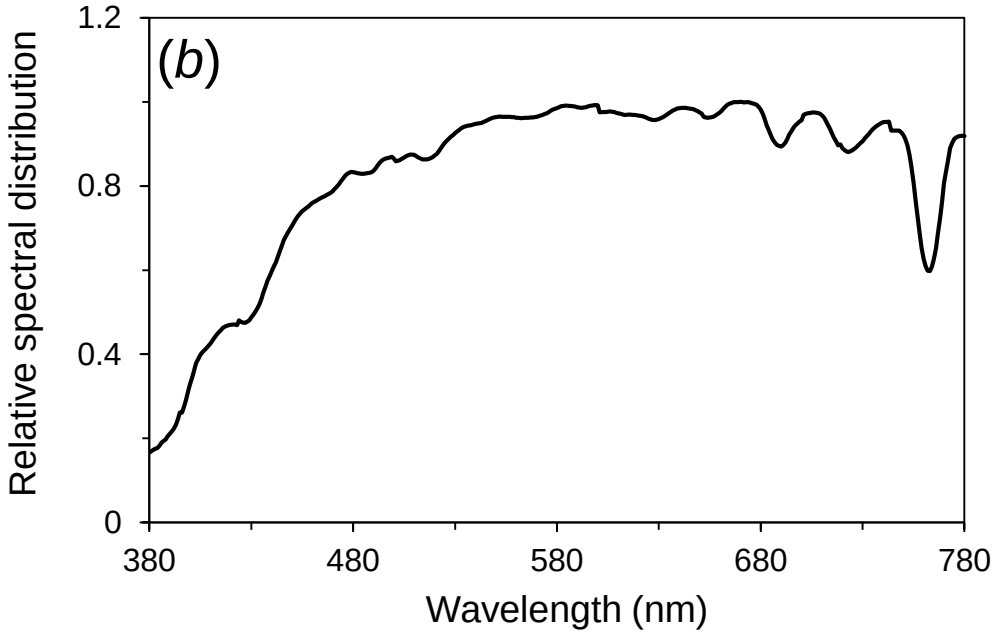
Fig. S8. The rate of DCIP photoreduction in the thylakoid membranes isolated from cucumber leaves after the exposure to full sunlight for 3 h. DCIP photoreduction was spectrophotometrically determined in the presence (+) and absence (−) of 1 mM DPC. Measurements were made using the same thylakoid preparations that were used in Figure 8.

Fig. S9. F_o, F_J, F_m, $(F_J - F_o)/F_J$ and F_v/F_m after the dark-RT or dark-chilling for 48 h with a direct excitation fluorometer PAR-FluoPen FP110/S (Photon Systems Instruments, Czech Republic). After the treatment, the leaves were vacuum-infiltrated in the dim light with 1% v/v DMSO (as a control) or 100 μM DCMU. The infiltrated leaves were kept in the dark for at least 20 min and then the OJIP-test was measured. F_J is the fluorescence intensity at J-step at 2 ms. $(F_J - F_o)/F_J$ and F_v/F_m in were calculated from the fluorescence levels obtained in the absence and presence of DCMU. Each bar represents the mean ± SD ($n \geq 15$). The Tukey-Kramer test, $P > 0.05$. According to Osmond et al. (2017), $(F_J - F_o)/F_J$ would reflect the Q_A-reducing activity better than F_v, because the latter is affected by the redox state of plastoquinone pool. However, $(F_J - F_o)/F_J$ after dark-chilling was not statistically different between water- and DCMU-infiltrated leaves, while F_v/F_m in DCMU-infiltrated leaves was greater than that in the water-infiltrated leaves. No differences in $(F_J - F_o)/F_J$ could be attributed to a high level of F_J in water-infiltrated leaves.

Fig. S10. Re-calculated $(F_J - F_o)/F_J$ and F_v/F_m after the dark-chilling for 48 h in DCMU-infiltrated leaves using the F_o levels obtained in water-infiltrated leaves. Each fluorescence parameter was derived from the Supplementary Fig. S9.

Fig. S11. Chlorophyll fluorescence transients after the dark-chilling treatment. Chlorophyll fluorescence transients induced by the SP (at 7000 μmol m^{-2} s^{-1}) recorded in the Fast Acquisition Mode of a PAM-2500 in DCMU- (light green) and water-infiltrated (grey) leaves after the dark-chilling treatment at 4˚C for 48 h. Arrows indicate the K-peak at about 300 μs, which has been claimed to be associated with heat stress and to indicate the inactivation of the OEC.





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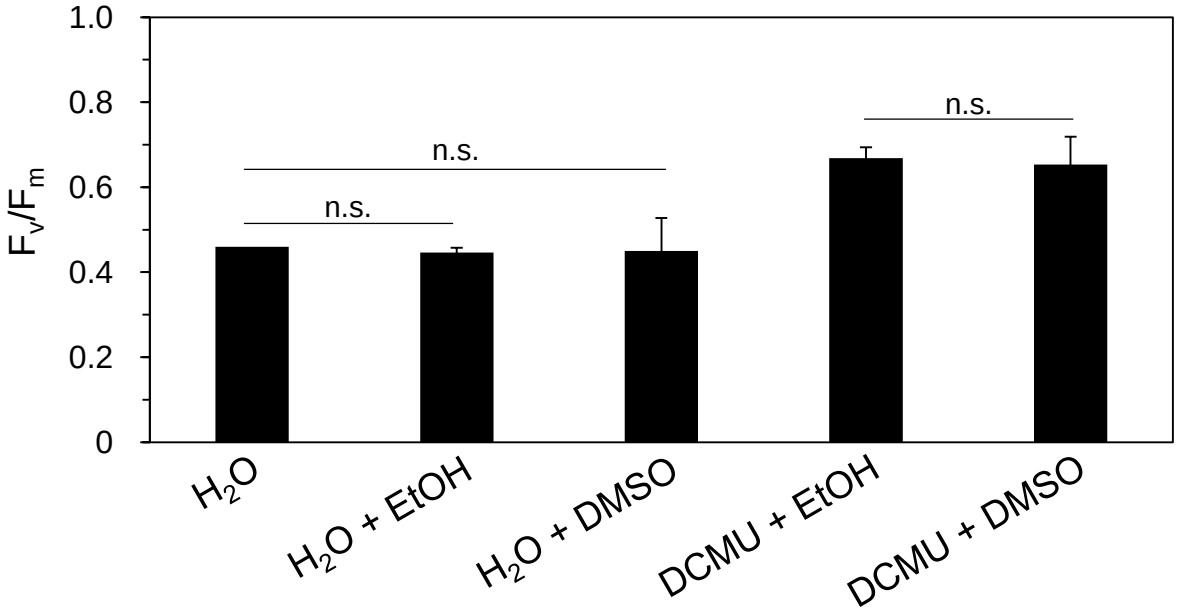

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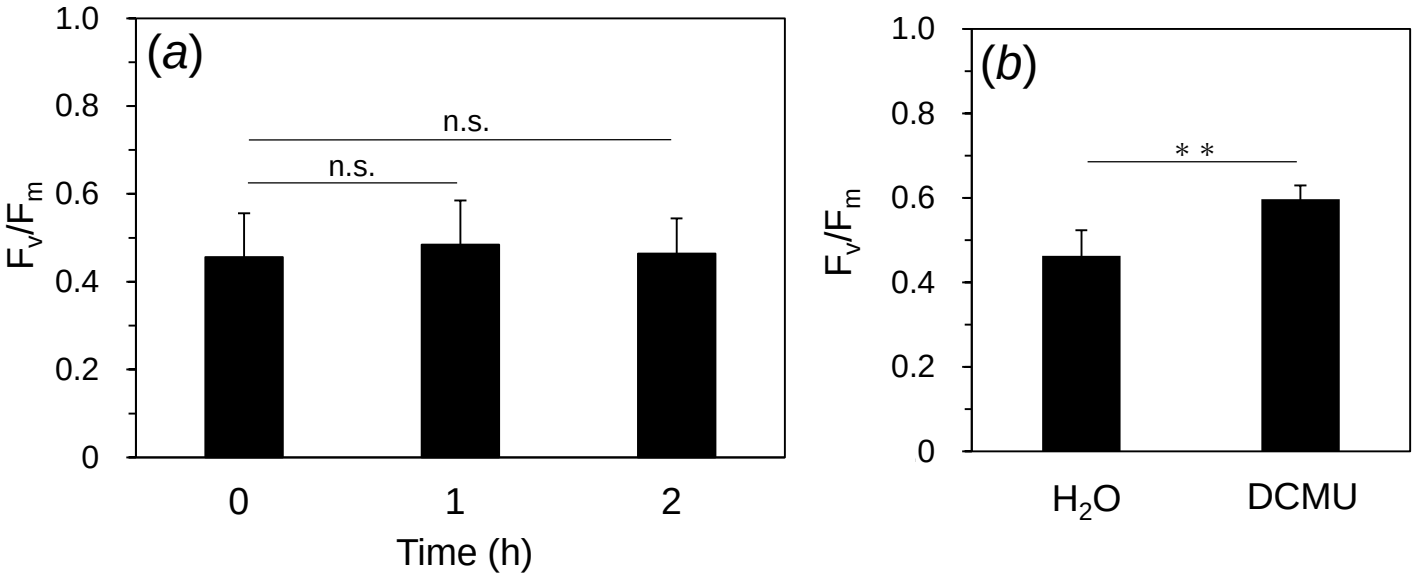

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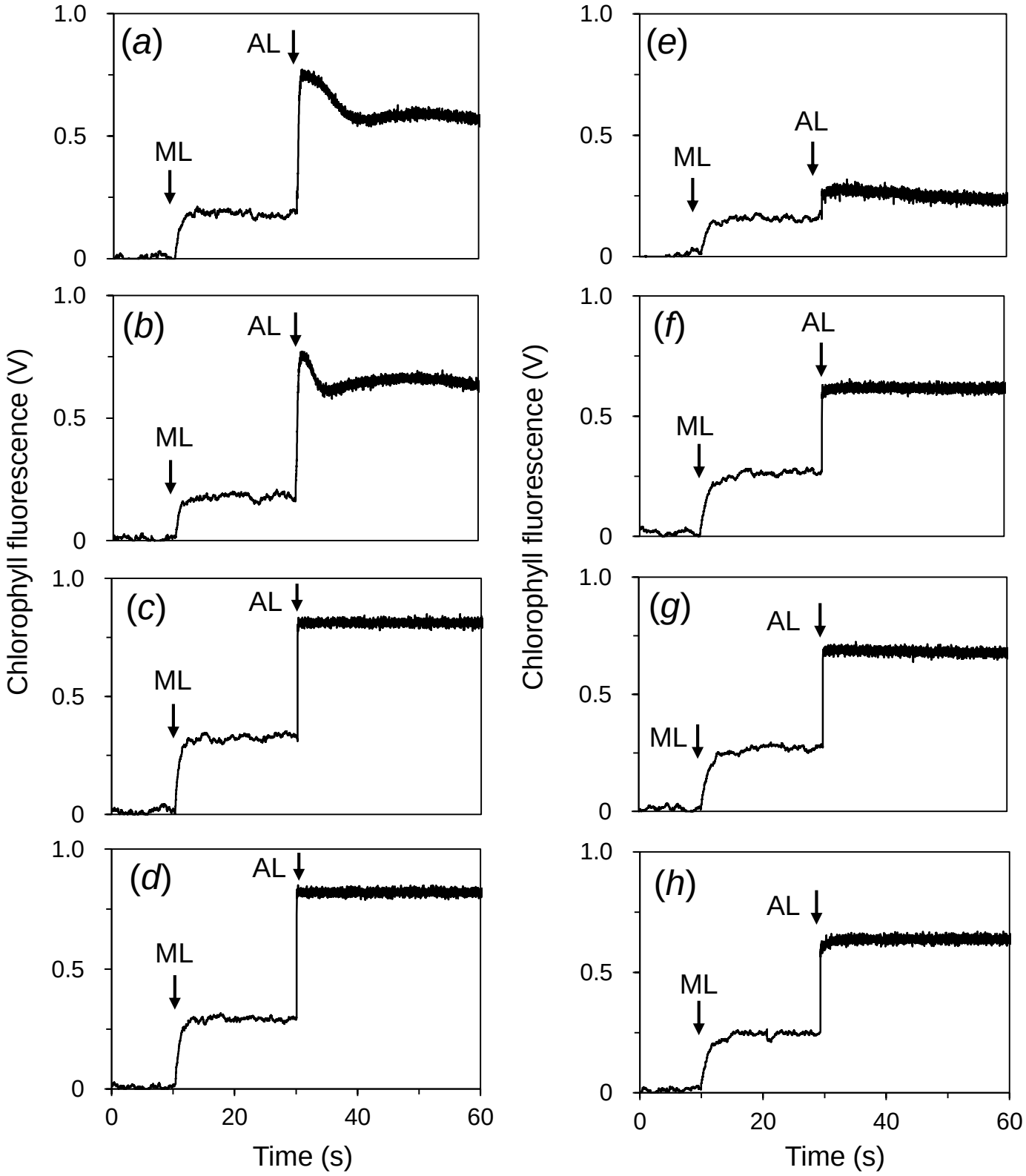
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
ML
AL
1.0
0.5
0
0
20
40
60
Time (s)
Chlorophyll fluorescence (V)

Supplementary Fig. S4. Changes in the chlorophyll fluorescence transient in cucumber leaves before and after the dark-chilling treatment for 48 h.

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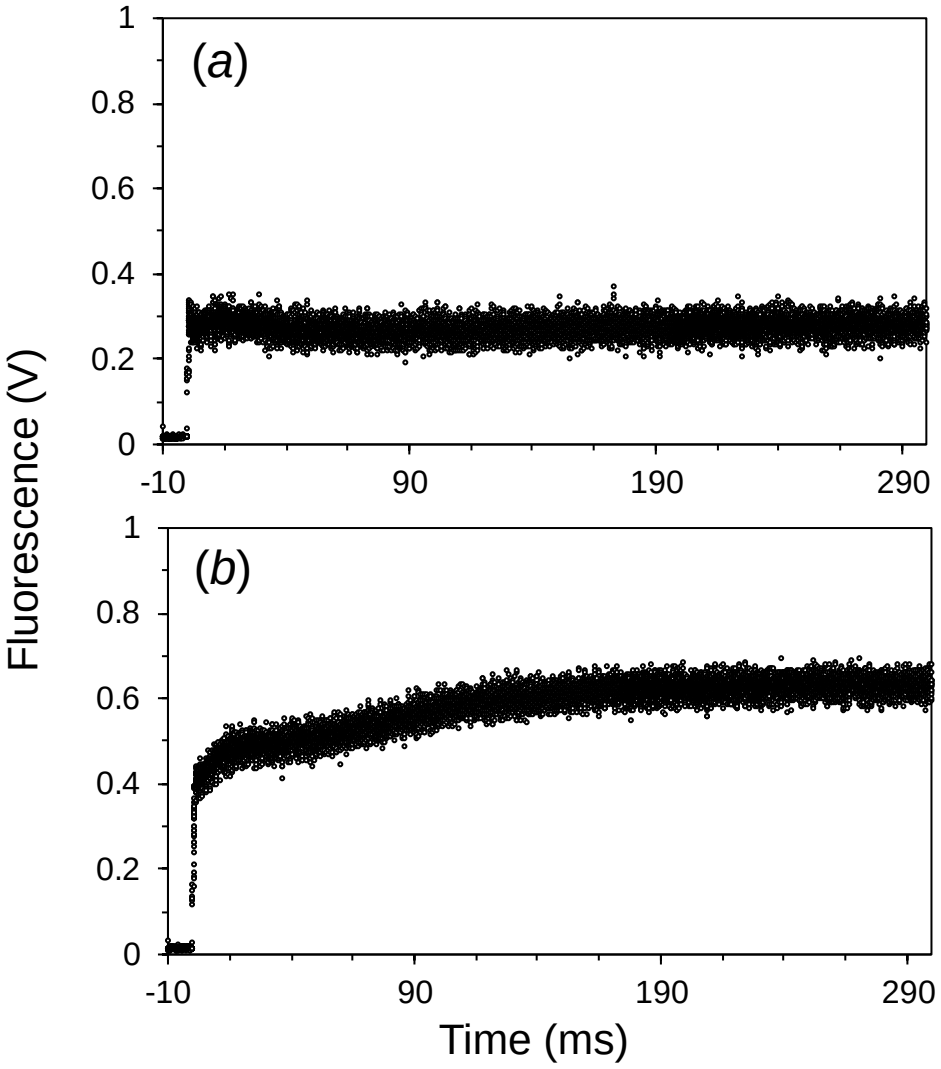

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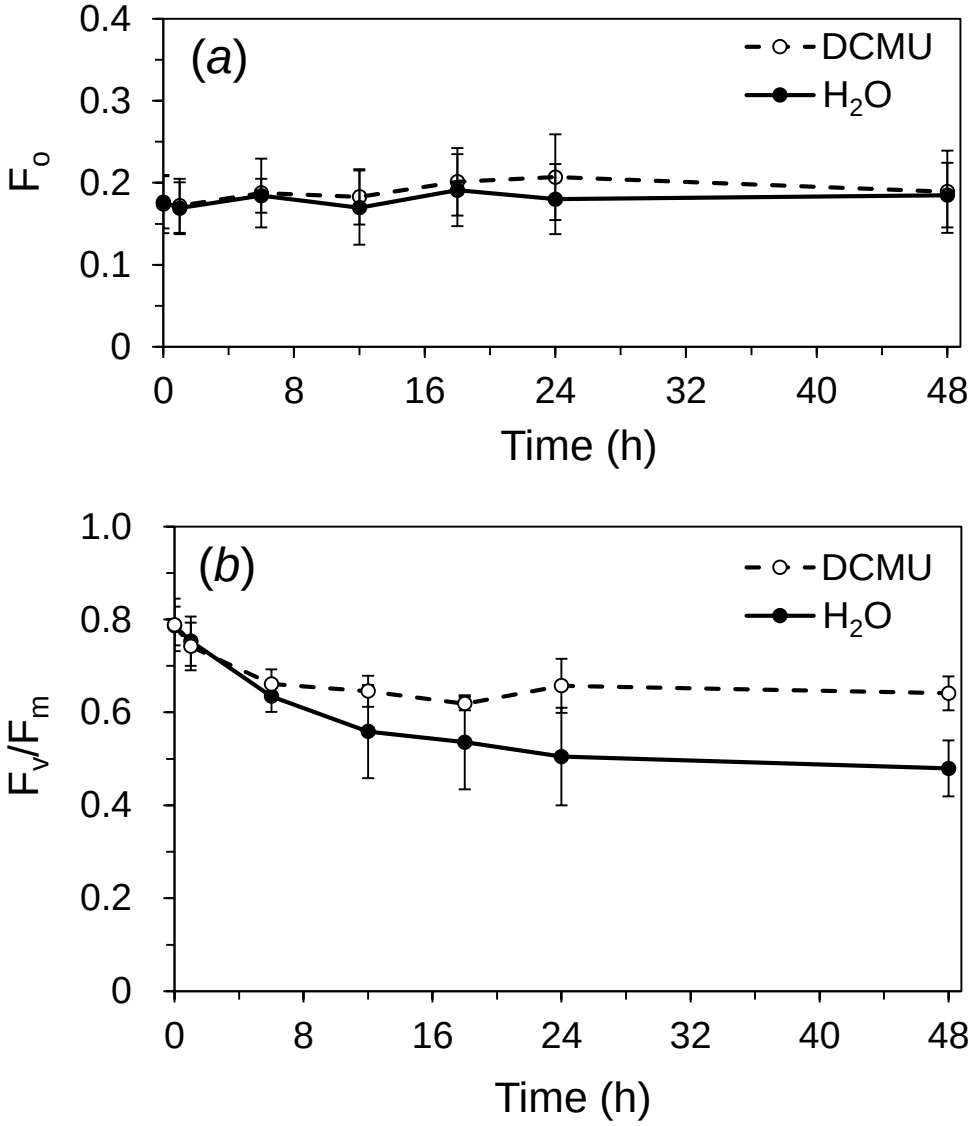

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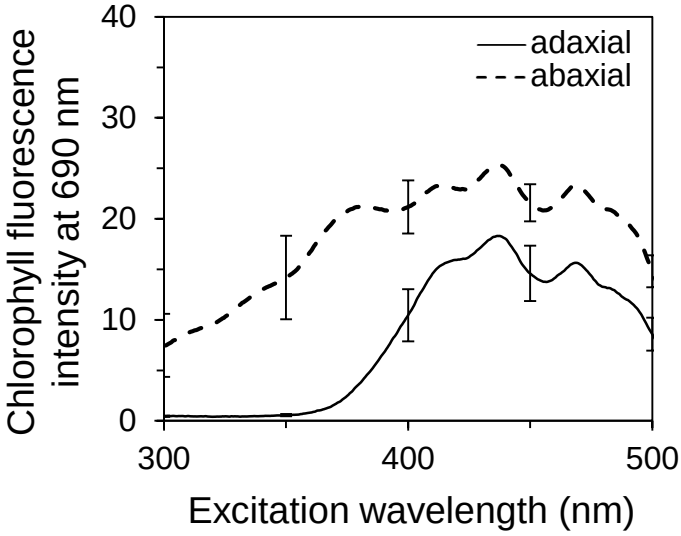

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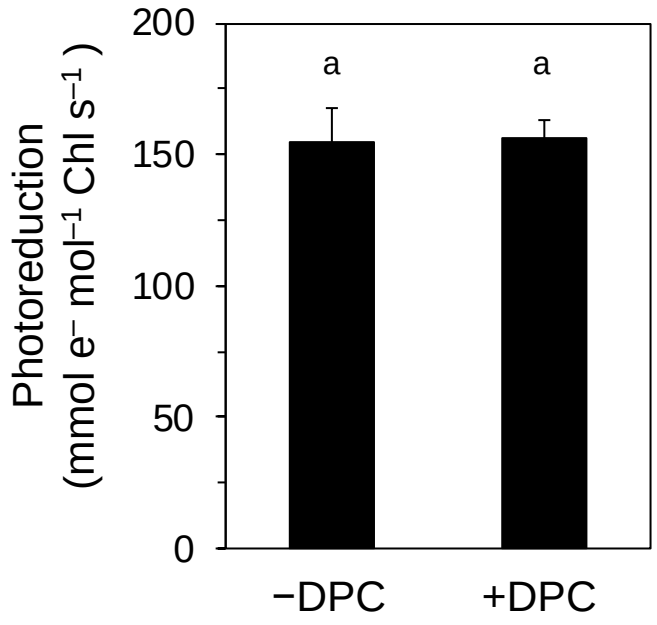

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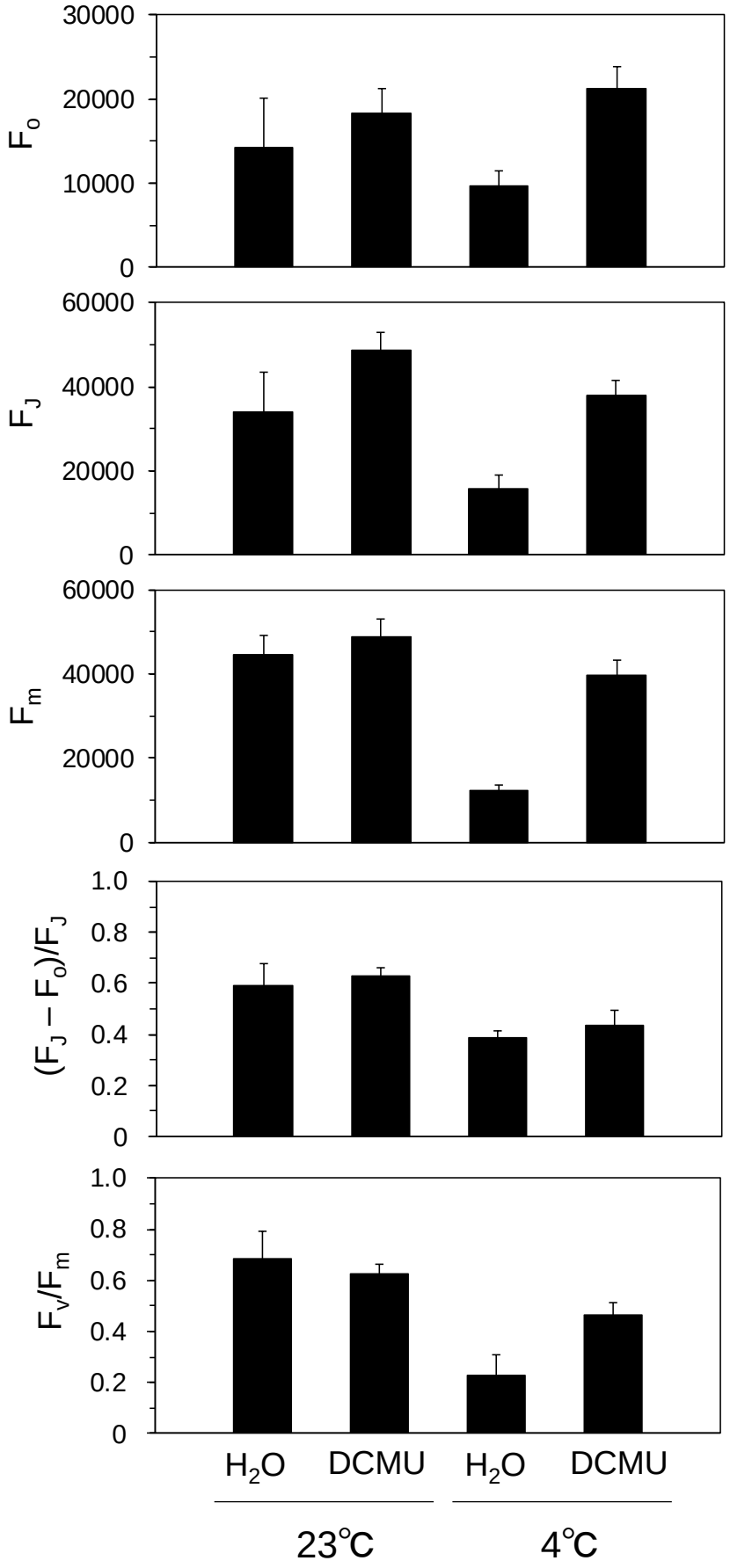
30000
20000
10000
0
F_o
60000
40000
20000
0
F_J
60000
40000
20000
0
F_m
1.0
0.8
0.6
0.4
0.2
0
$(F_J - F_o)/F_J$
1.0
0.8
0.6
0.4
0.2
0
F_v/F_m
H_2O
DCMU
H_2O
DCMU
23℃
4℃

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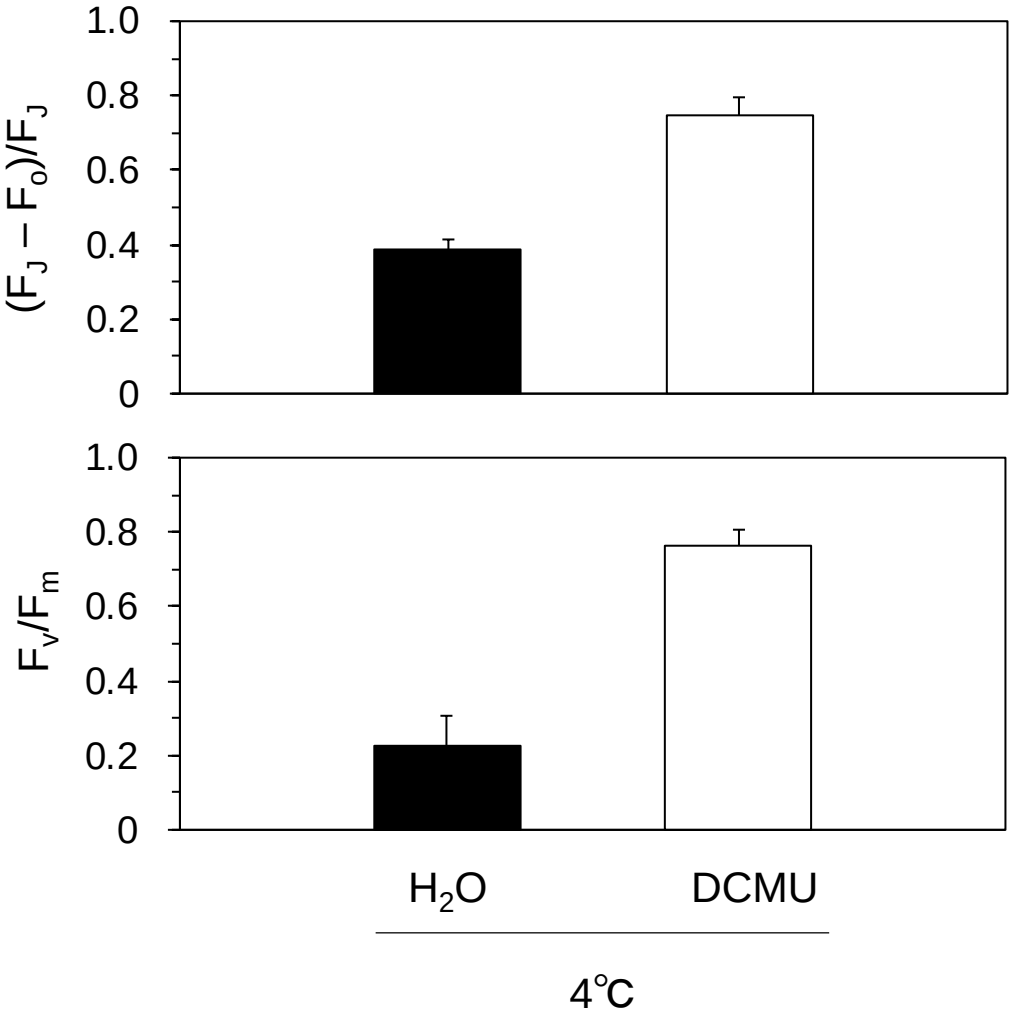

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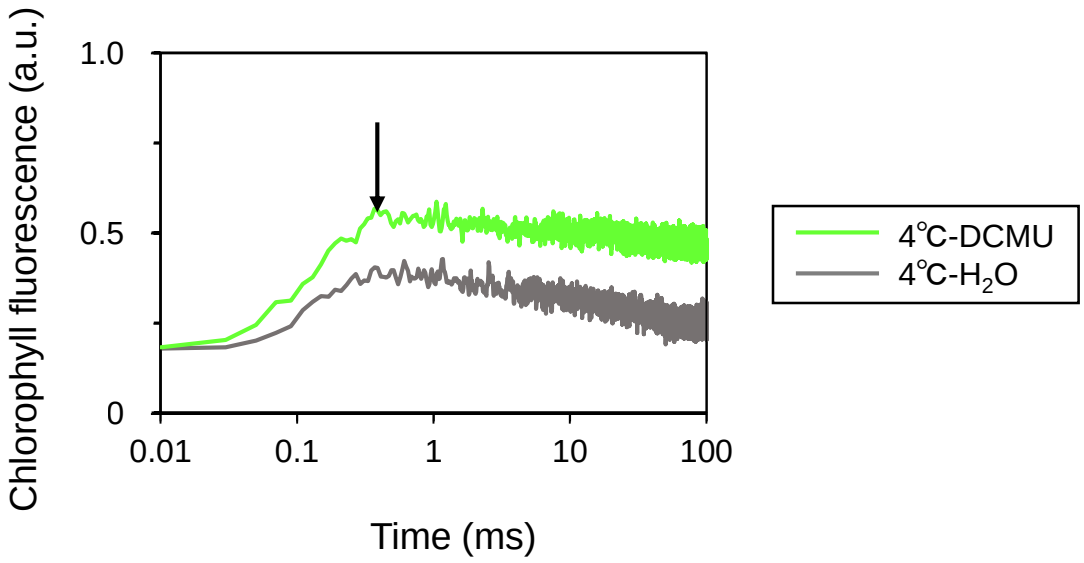

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