Plants switch photosystem at high temperature to protect photosystem II

Yasuo Yamauchi[1], Yoko Marutani[1], Seiji Akimoto[2], Yukihiro Kimura[3], Masaharu Mizutani[1] & Yukihiro Sugimoto[1]

Plants are often exposed to temperatures of around 40 °C. These temperatures can cause serious damage to photosystems, yet plants can survive with minimum damage. Here, we show that plants switch photosystem to protect photosystem II (PSII) at 40 °C. Using wheat and Arabidopsis seedlings, we investigated the mechanisms of heat-derived damage in the dark and avoidance of damage in the light. Heat treatment at 40 °C in the dark caused serious damage to PSII: the maximum quantum yield of PSII (Fv/Fm) and oxygen evolution rapidly decreased. The damage was due to the degradation of the D1 protein (shown by immunochemical analysis) and the disturbance of energy transfer in PSII core chlorophyll-binding proteins CP43 and CP47 (shown by time-resolved fluorescence measurement). The damage to PSII might be attained to enhanced introduction of electrons from the reducing power of the stroma into thylakoid membranes, causing subsequent electron backflow to PSII. Plants treated at 40 °C in the light avoided PSII damage and showed preferential excitation of photosystem I (PSI), phosphorylation and migration of light-harvesting complex II (LHCII), which indicate state transition of the photosystem to enhance thermal dispersion and light-driven cyclic electron flow around PSI. These results suggest that heat damage to PSII is probably due to a backflow of reducing power from the stroma to PSII, and that light causes a state transition of photosystem, driving cyclic electron flow and thus protecting PSII from damage.

As global temperatures rise, plants are becoming exposed to high temperatures more often. For most plant species, temperatures above 40 °C are perilous. Photosynthetic activity was decreased at 40 °C: the maximum quantum yield of PSII (Fv/Fm) in seedlings of various species decreased rapidly at 40 °C in the dark within an hour (Fig. 1a). Young seedlings tended to be more susceptible, and the dicots tobacco, tomato and *Brassica rapa* were damaged at 42.5 °C but not at 40 °C (Supp. Fig. 1). In the case of wheat seedlings, the most susceptible plant tested, heat treatment at 40 °C in the dark for 30 min (D/40-°C/30-min) decreased Fv/Fm from 0.75 to 0.5. This damage was strictly temperature dependent: Fv/Fm did not decrease at or below 35 °C (Fig. 1b), as also seen in Arabidopsis (Fig. 1c), and the decrease of Fv/Fm was due to decrease of Fm but not Fo (Supp. Fig. 2). However, such

damage is not usually realized in nature, because high air temperatures in daytime coincide with light, which cancels the decrease of Fv/Fm (Figs. 1a–c). Supporting these results, oxygen evolution by thylakoid membranes prepared from D/40-°C/30-min-treated wheat leaves was reduced, but that from leaves heat-treated in the light was not (Fig. 1d).

Time-resolved fluorescence spectra (TRFS) measurements revealed that the D/40-°C/30-min treatment disturbed energy transfer in PSII (Fig. 2a). In TRFS of both control and D/40-°C/30-min-treated wheat leaves, a peak around 680 nm at the very beginning due to PSII gave way to a peak around 735 nm due to PSI red-Chl[1]. The difference between the spectra showed a disturbance of energy transfer in PSII, because the peaks around 685 and 695 nm were due to energy transfer from CP43 and CP47, respectively, to the PSII reaction centre [2]. In contrast, PSI fluorescence, which peaked around 735 nm, did not change by heat treatment in the dark.

We used immunochemical analysis to examine damage to proteins involved in photochemical reactions. Protein profiles of control plants and D/40-°C/30-min-treated plants were apparently not different (Fig. 2b, CBB staining). But among major photochemical proteins tested, D1 was damaged by D/40-°C/30-min treatment (Fig. 2b, Immunoblotting). Damage of D1 protein was also observed in Arabidopsis (Fig. 2c). As D1 plays a central role in photochemical reactions in PSII [3], damage to it influences PSII activities such as Fv/Fm and oxygen evolution. Degradation of D1 as a main cause of the D/40-°C inactivation was also supported by the results that recovery from the damage was a light-dependent process (Supp. Fig. 3), and was delayed by treatment with two inhibitors of protein translation, cycloheximide (Chx, inhibitor of the synthesis of nucleus-encoded proteins) and chloramphenicol (Cp, inhibitor of the synthesis of chloroplast-encoded proteins) in Arabidopsis, because synthesis of D1 is light dependent [4] and involves both cytosolic and chloroplastic translation machineries [5]. On the other hand, D/40-°C/30-min treatment disturbed the transfer of light energy through CP43 and CP47, but not the contents of CP43 and CP47, suggesting that the treatment disturbed the configuration or conformation of energy transfer molecules that bind to CP43 and CP47. Other proteins located in cytochrome (cyt) b_6f and PSI were not changed. As the results of Fv/Fm and oxygen evolution showed, damage to D1 was avoided in the presence of light (Fig. 2b, lane L40°C).

How is PSII damaged? To answer this question, we hypothesized the involvement of a backflow of electrons held in the reducing power of the stroma to the PSII core, because PSI-centred cyclic electron flow (CEF)-mediated incorporation of stromal reducing power was activated at 40 °C (light-independent transient increase of fluorescence just after actinic light was turned off: Fig. 3a) but not at or below 35 °C (Supp. Figs. 4, 5). The light-independent

reduction of plastoquinone (PQ) was almost equal to the light-dependent reduction of PQ through PSII at 40°C, and the following decrease of fluorescence was rapid (Fig. 3a), suggesting that PQ reduction (i.e. incorporation of stromal reducing power) and subsequent PQ re-oxidation (i.e. flow into other components) were activated at 40 °C. In the dark, the incorporation of stromal reducing power into PQ in thylakoid membranes is thought to be mediated by ferredoxin (Fd)-dependent route[6]. To assess the contribution of the route to the damage, we used two T-DNA–tagged Arabidopsis mutants, an NAD(P)H dehydrogenase (NDH)-deficient mutant (*ndh-o*)[7] and a leaf-type Fd-NADPH reductase–deficient mutant (*lfnr*) [8] , in both of which PSII showed higher tolerance to D/40-°C/30-min treatment than the wild-type, Col-0: i.e. the decrease of Fv/Fm in the mutants was smaller (Fig. 3b). Likewise, the light-independent PQ reduction curve indicated that reduction and subsequent re-oxidation of PQ were slower in both mutants than in Col-0 (Fig. 3c). This light-independent PQ re-oxidation is interpreted to reflect a backflow of electrons from PQ to PSII, as characterized by thermoluminescence around 40–50 °C, which is assigned to "afterglow" emission due to recombination of $S_{2(3)}Q_BX^-$ species derived from the stromal reducing power via CEF [9-11]. Thus, excited energy generated by $S_{2(3)}Q_BX^-$ recombination might be responsible for damage to primary D1, resulting in deterioration of PSII activity; this is partly similar to the mechanism of acceptor-side photoinhibition[12].

As described above, light cancels the decrease of Fv/Fm, the loss of oxygen evolution and the degradation of D1 protein caused by treatment at 40 °C. In the case of wheat, light was essential for survival at 40°C (Supp. Fig 3). Chlorophyll induction curves indicate that even under moderate light intensity (190 μmol photon m^{-2} s^{-1}), most light energy was quenched by non-photochemical reaction in wheat leaves treated at 40 °C, whereas most light energy was used by photochemical reaction at 25 °C (Fig. 3a). Detailed quench–relax analysis of a wheat leaf treated at 40 °C for 30-min in the presence of light (L/40-°C/30-min) suggested that the enhanced non-photochemical quenching (NPQ) was divided into two components, fast ($_fq_r$) and medium ($_mq_r$) quench–relaxes (Fig. 4a). NPQ is attributed to three processes (energy dispersion via the xanthophyll cycle, state transition and photoinhibition), and $_fq_r$ and $_mq_r$ are assigned as energy dispersion via the xanthophyll cycle and state transition, respectively [13]. Phosphorylation of thylakoid proteins, a well known biochemical indicator of state transition, was detected by immunoblotting using anti-phosphothreonine monoclonal antibody in both wheat and Arabidopsis leaves treated with L/40-°C/30-min (Fig. 4b; Supp. Fig. 6). Major phosphorylated protein observed in wheat was determined as LHCb1 by proteomic analysis (Supp. Fig. 7) Isolated PSI supercomplex from the L/40-°C/30-min

sample had more LHCII proteins (LHCb1, LHCb2, CP29, CP26), especially the inner antenna monomeric CP29 and CP26, than the control, (Fig. 4c). This result possibly reflects the structure of the binding site in PSI, which is predicted to prefer monomeric LHCII [14]. Corresponding to this result, TRFS of the L/40-°C/30-min sample (Fig. 4d) differed from that of the control sample (Fig. 2a, left panel). A large peak of PSI Chl fluorescence around 735 nm was immediately detected after Chl excitation, whereas PSII fluorescence of the L/40-°C/30-min sample was lower at the very beginning of Chl excitation and then became almost the same as the control, suggesting that a state of PSI is state 2 where PSI is preferentially excited by light energy, probably via mobilized LHCII proteins. In contrast, no thylakoid protein was phosphorylated in D/40-°C/30-min wheat (Supp. Fig. 8), coinciding with the results of TRFS that showed photosystem in D/40-°C/30-min wheat remained state 1 (Fig. 2a).

State transitions have been extensively studied in *Chlamydomonas reinhardtii*, and their impact on energy balancing between photosystems and the promotion of CEF around PSI is well established[15,16]. In higher plants, state transition at higher temperature has been observed by earlier studies[17-19], however the physiological significance of state transitions is less clear because their mobile LHCII pools are significantly smaller than those in cyanobacteria and green algae [20]. Our detailed study indicates that state transition of the photosystem might play an important role in heat stress response even though quantitative change is low: i.e. it provides rapid electron flow from plastohydroquinone (PQH_2) to PSI to protect against backflow of electrons to PSII, presumably achieved by oxidation of downstream components (e.q. cyt b_6f and plastocyanin) by light-driven PSI. This mechanism would compatibly contribute to prevent PSII photoinhibition via over-reduction of PQ occurring under excess light condition[12]. Induction of state transition at higher temperatures is plausibly triggered by enhanced PQH_2 reduction, which induces state transition via activation of Ser–Thr protein kinase [15,21]. Activated CEF acidifies the lumen, resulting in enhanced heat dissipation via the xanthophyll cycle, and promotes the supply of the abundant ATP needed for the maintenance of intracellular components such as heat-shock proteins, ATP-dependent proteases[12] and chaperonines[22], which catalyse degradation and refolding through the use of ATP as chemical energy without the accumulation of excess NADPH.

A series of pioneering studies pointed out the importance of the relationships among temperature, light condition and photosynthetic electron flow for determining damage to photosystems at high temperatures [23,24]. Here, we show that the electron flow in the photosystems plays a critical role in the heat stress response of plants. Normally, electrons

taken from water molecules in PSII flow via linear electron flow, known as the Z scheme, to produce NADPH (Fig. 4e, top). However, at 40 °C without light, PSII (especially D1 protein) is faced with damage due to backflow of the reducing power from the stroma, because light-driven electron flow is absent (Fig. 4e, middle). Therefore, the plant switches its active photosystem via state transition to alleviate the damage to PSII even under low light (Fig. 4e, bottom), as well as to avoid overexcitation and destruction of PSII under high light intensities during the course of the day [25].

Methods summary

Plants were grown in a growth chamber (12 h light, 80-100 μmol photon m^{-2} s^{-1}, 25 °C). Plants were heat-treated by incubation at desired temperature under fluorescent light (100 μmol photon m^{-2} s^{-1}) or in the dark (Biotron LH-220S, Nippon Medical & Chemical Instruments Co. Ltd., Tokyo, Japan). Fv/Fm and oxygen evolution were measured as described previously [26]. Time-resolved fluorescence spectra (TRFS) were measured with a picosecond-time-correlated single-photon counter [27]. The Arabidopsis T-DNA–tagged mutants *lfnr* (SALK_067668C) and *ndh-o* (SALK_068922) were obtained from ABRC [28]. PSI was isolated as described [29] with slight modifications (see Methods). Protein analysis used specific antibodies[26,30].

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Author contributions Y.Y. and Y.M. performed physiological and biochemical analyses, and M.M. and Y.S. supervised. Y.K. and Y.M. measured oxygen evolution. S.A. performed TRFS analysis. Y.Y. wrote the paper, and all of the authors joined the discussion of the results. The whole study was designed by Y.Y. Correspondence and requests for materials should be addressed to Y.Y. (yamauchi@kobe-u.ac.jp).

[1] Graduate School of Agricultural Science, Kobe University

[2] Graduate School of Science, Kobe University

[3] Organization of Advanced Science and Technology, Kobe University

Figure legends

Figure 1| PSII function is damaged by heat treatment at 40 °C in the absence of light, but not in the presence of light. a, One-week-old seedlings were heat-stressed with (white) or without (black) light (100 μmol photon m^{-2} s^{-1}). **b,** Wheat seedlings were grown in light at 25, 35, 40 or 45 °C. **c,** Arabidopsis seedlings were grown in light at 25, 35, 40 or 45 °C. **d,** Oxygen evolution by thylakoid membranes prepared from wheat seedlings heat-stressed at 40 °C for 30 min with or without light. All error bars indicate ±SEM (n = 3).

Figure 2| Disturbance of energy transfer in CP43 and CP47, and degradation of D1 protein. a, TRFS analysis using leaf discs: left panel, control plant; middle panel, plant heat-stressed in the dark; right panel, spectral difference (stress minus control). Shaded regions represent fluorescence derived from PSII (pink) and PSI (blue). **b,** Profiles of major thylakoid proteins prepared from wheat seedlings heat-stressed at 40 °C for 30 min with or without light. After separation by SDS-PAGE, proteins were stained with CBB R-250 (left), or major photosynthetic proteins were detected by immunoblotting with specific antibodies (right). Fv/Fm of samples were 0.75 (control), 0.55 (D40°C) and 0.72 (L40°C). **c,** Immunochemical detection of D1 and PsaD proteins prepared from Arabidopsis seedlings heat-stressed at 40 °C for 30 min with or without light. Fv/Fm of samples were 0.75 (control), 0.55 (D40°C) and 0.72 (L40°C).

Figure 3| Enhanced introduction of stromal reducing power into PQ. a, Chlorophyll induction curve of wheat leaf discs at 25 and 40 °C. Arrows show switching on (↓) and off (↑) of measuring light (ML) and actinic light (AL). Open arrowheads show timing of saturated light pulse. NP, non-photochemical PQ reduction; P, photochemical PQ reduction. a.u., arbitrary units. **b,** PSII activity of Arabidopsis seedlings heat-stressed at 40 °C in the dark. Error bars indicate ±SEM (n = 3). **c,** (top panel) Schematic diagram of PQ reduction by stromal NADPH via Fd-dependent route on thylakoid membranes. Fd-PQ reductase (FQR) is assumed to be present in the Fd-dependent route, but not yet identified. (middle panel) Chlorophyll fluorescence induction curve of Arabidopsis at 40 °C. (bottom panel) Enlargements of light-independent PQ reductions boxed in middle panel. Fluorescence intensity is normalized to Fo value. Fs, steady level of chlorophyll fluorescence.

Figure 4| State transition of photosystem is a heat stress response. a, Quench–relax analysis of a wheat leaf heat-stressed at 40 °C for 30 min in light (100 μmol photon m^{-2} s^{-1}). Timing and intensity of actinic light (AL) and saturated pulse (SP) are as in Fig. 3a. **b,** Phosphorylation of threonine residue(s) of thylakoid proteins. Thylakoid membrane proteins

prepared from control (0) and L/40-°C/30-min (L40) samples were subjected to SDS-PAGE (CBB). Phosphorylation of Thr was detected by immunoblotting with anti-phospho-Thr antibody (anti-pThr). **c,** Increased amount of LHCII proteins in PSI isolated from L/40-°C/30-min sample. PSI isolated by sucrose-density-gradient centrifugation from control (C) and L/40-°C/30-min sample (L40) was subjected to SDS-PAGE (CBB). LHCII proteins were detected by immunoblotting with anti-LHCII protein antibodies (Immunoblotting). Migration of phosphorylated protein was identical to LHCb1 and b2. **d,** TRFS of L/40-°C/30-min wheat sample. Shaded regions represent fluorescence derived from PSII (pink) and PSI (blue). Dotted box shows rapid PSI excitation probably due to migrated LHCII. **e,** Schematic diagrams of electron flow (grey arrows) under three different conditions. Under normal condition (top), electrons flow linearly from PSII to PSI. At 40 °C without light (middle), backflow of electrons from stromal reducing power possibly damages PSII, mainly D1 protein. At 40 °C with light (bottom), PSII is protected by enhanced CEF via energy dispersion mediated by the xanthophyll cycle and light-driven electron flow. Green columns, inner LHCII proteins (CP29 and CP26); white columns, outer LHCII proteins (LHCb1 and b2).

Methods

Plant materials, heat treatments, and measurement of chlorophyll fluorescence and oxygen evolution

Plants were grown in a growth chamber (12 h light, 80-100 μmol photon m^{-2} s^{-1}, 25 °C). One week old-Plants (2-3 week old-plants in the case of Arabidopsis) were heat-treated by incubation at 40 °C under fluorescent light (100 μmol photon m^{-2} s^{-1}) or in the dark (Biotron LH-220S, Nippon Medical & Chemical Instruments Co. Ltd., Tokyo, Japan). After dark-adaptation, Fv/Fm was measured with a pulse-modulated fluorometer (Junior-PAM, Heinz Walz GmbH, Effeltrich, Germany)[26]. Oxygen evolution was measured at 25 °C with a Clark-type oxygen electrode (Rank & Brothers Ltd., UK) in 50 mM MES-NaOH (pH 6.0), 5 mM $MgCl_2$ and 15 mM NaCl (MMN buffer) containing 5 μM $MnSO_4$, 5 mM $CaCl_2$, 0.4 M sucrose and 0.5 mM *p*-phenyl-benzoquinone as an electron acceptor. Chemicals used were generally of reagent grade (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The Arabidopsis T-DNA–tagged mutants *lfnr* (SALK_067668C) and *ndh-o* (SALK_068922) were obtained from ABRC [28]. Loss of the coded proteins was confirmed by immunoblotting using anti-leaf–type FNR (a generous gift of Prof. Hase, Osaka University) and anti-ndh-o antibody (Supp. Fig. 9).

Time-resolved fluorescence spectra analysis

TRFS were measured with a picosecond-time-correlated single-photon counter [27]. The light source was a Ti:sapphire laser (Coherent MIRA 900, USA) emitting at 400 nm, which excites both Chl *a* and Chl *b*. We used a microchannel plate photomultiplier (Hamamatsu R3809, Japan) as a detector, combined with a monochromator (P-250, Nikon, Japan). The time step was 2.6 or 52 ps/channel. The laser intensity was set to give fluorescence signals of <10 000 counts/s around the fluorescence peak wavelengths, in which condition the samples did not suffer damage, at a repetition rate of 2.9 MHz. All measurements were carried out at −196 °C with a custom-made Dewar system.

Chlorophyll fluorescence analysis

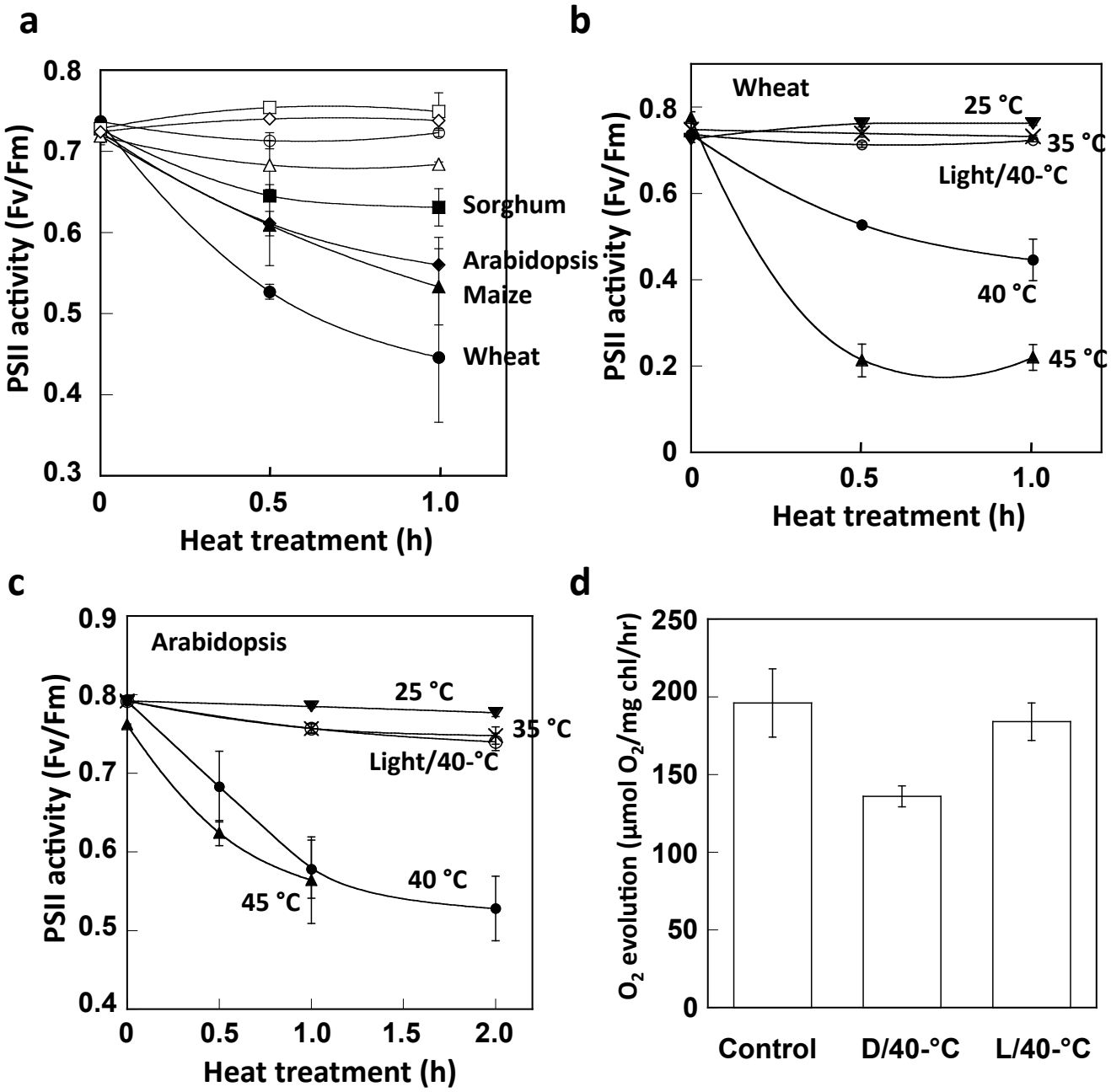
A leaf detached from a dark-adapted plant was placed on wet paper whose temperature was controlled by a heat block. After 5 min temperature equilibration, chlorophyll fluorescence was measured by Junior-PAM and analysed with WinControl v. 3.1 software (Walz).

Isolation of PSI

PSI was isolated by the method of Croce et al. [29] with slight modifications. Isolated thylakoid membranes solubilized with 0.8% (w/v) dodecyl-D-maltoside in MMN buffer were loaded onto a sucrose gradient (0.4–1.3 M sucrose in MMN buffer containing 0.05% [w/v] dodecyl-D-maltoside). After ultracentrifugation at 183 000 ×*g* for 7 h at 4 °C, the lower green band was recovered and used as the PSI fraction.

Protein analysis

Thylakoid membrances were prepared as a method described previously[26]. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system in 12% or 15% (w/v) acrylamide gel[26,30]. The separated proteins were electroblotted onto polyvinylidene fluoride membrane (ATTO Corp., Tokyo, Japan) according to the manufacturer's instructions. For detection of oxygen-evolving-complex 33-kDa protein (OEC33) and leaf-type FNR (LFNR), we used anti-OEC33 and anti-LFNR polyclonal antibodies (a generous gift of Prof. Enami, Tokyo University of Science, Japan, and Prof. Hase, Osaka University, Japan, respectively) as the primary antibody. Other antibodies were obtained from Agrisera (Vännas, Sweden). Alkaline phosphatase–conjugated anti-rabbit or anti-mouse IgG antibody (Sigma-Aldrich) was used as a secondary antibody. Signals were visualized by bromo-chloro-indolyl phosphate and nitroblue tetrazolium. Phosphorylated proteins were purified by ProQ Diamond Phosphoprotein Enrichment Kit (Invitrogen, CA, USA).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PSII activity (Fv/Fm)
Heat treatment (h)
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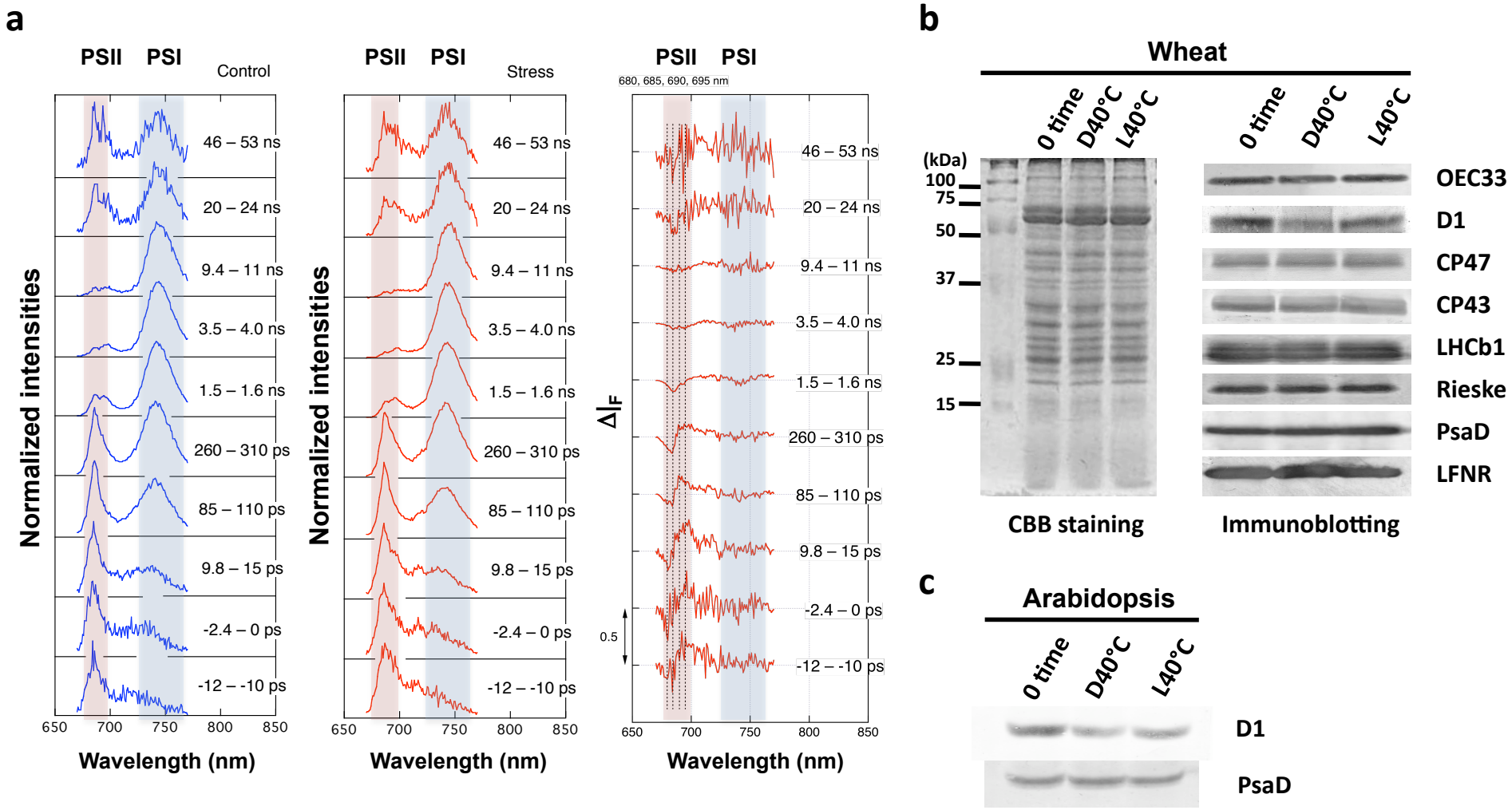
40 °C
45 °C
PSII activity (Fv/Fm)
Heat treatment (h)
c
Arabidopsis
25 °C
35 °C
Light/40-°C
40 °C
45 °C
PSII activity (Fv/Fm)
Heat treatment (h)
d
O2 evolution (µmol O2/mg chl/hr)
Control
D/40-°C
L/40-°C

Fig. 1.


a
PSII
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Control
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680, 685, 690, 695 nm
46 – 53 ns
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9.4 – 11 ns
3.5 – 4.0 ns
1.5 – 1.6 ns
260 – 310 ps
85 – 110 ps
9.8 – 15 ps
-2.4 – 0 ps
-12 – -10 ps
Normalized intensities
ΔIF
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700
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L40°C
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100
75
50
37
25
15
CBB staining
Immunoblotting
OEC33
D1
CP47
CP43
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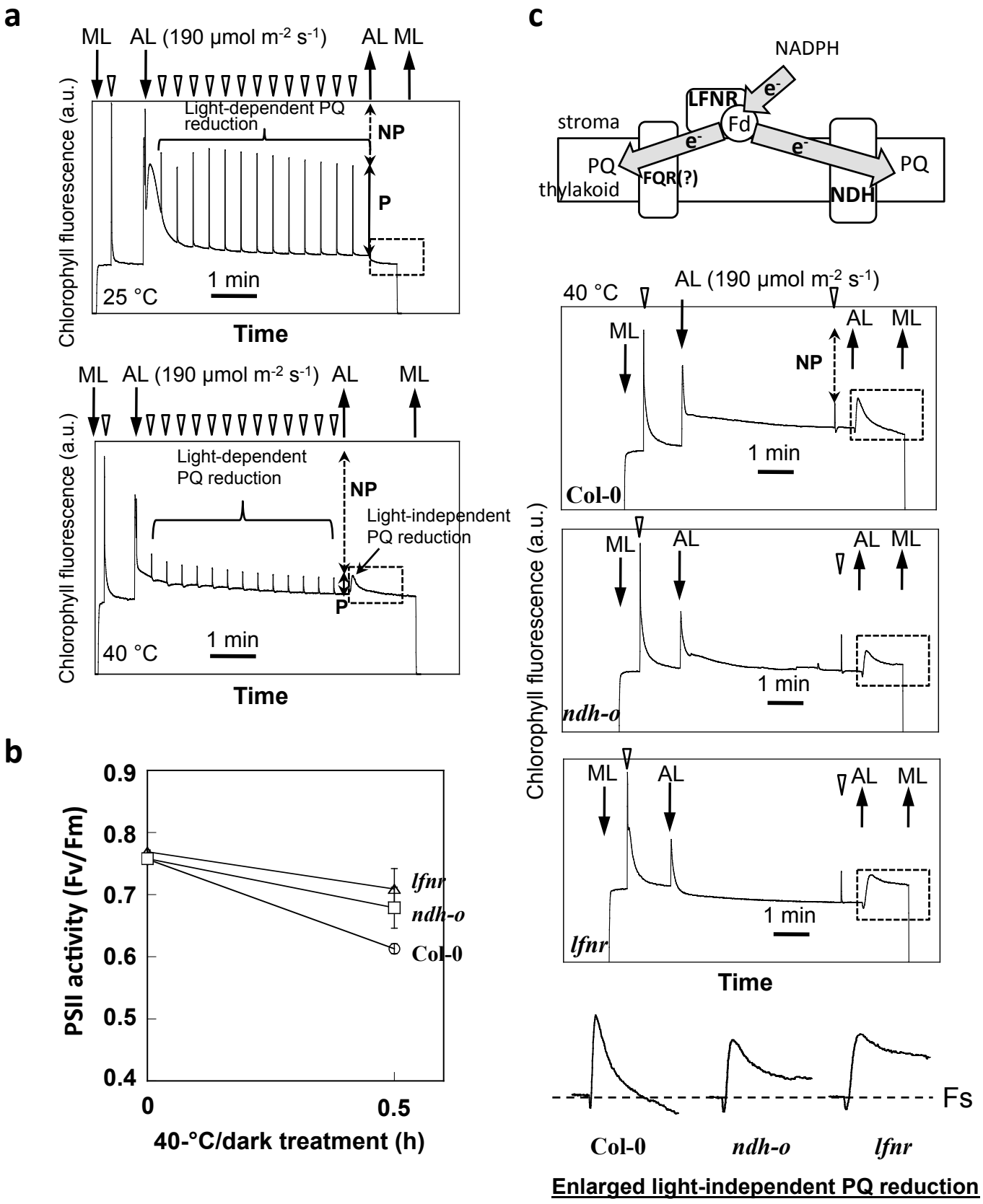
Fig. 2


a
ML
AL (190 μmol m-2 s-1)
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ML
AL (190 μmol m-2 s-1)
AL
ML
Light-dependent PQ reduction
NP
Light-independent PQ reduction
P'
40 °C
1 min
Chlorophyll fluorescence (a.u.)
Time
b
PSII activity (Fv/Fm)
0.9
0.8
0.7
0.6
0.5
0.4
0
0.5
40-°C/dark treatment (h)
lfnr
ndh-o
Col-0
c
NADPH
LFNR
e-
Fd
stroma
PQ
FQR(?)
thylakoid
NDH
PQ
40 °C
AL (190 μmol m-2 s-1)
ML
AL
ML
NP
1 min
Col-0
ndh-o
lfnr
Chlorophyll fluorescence (a.u.)
Time
Fs
Col-0
ndh-o
lfnr
Enlarged light-independent PQ reduction

Fig. 3.

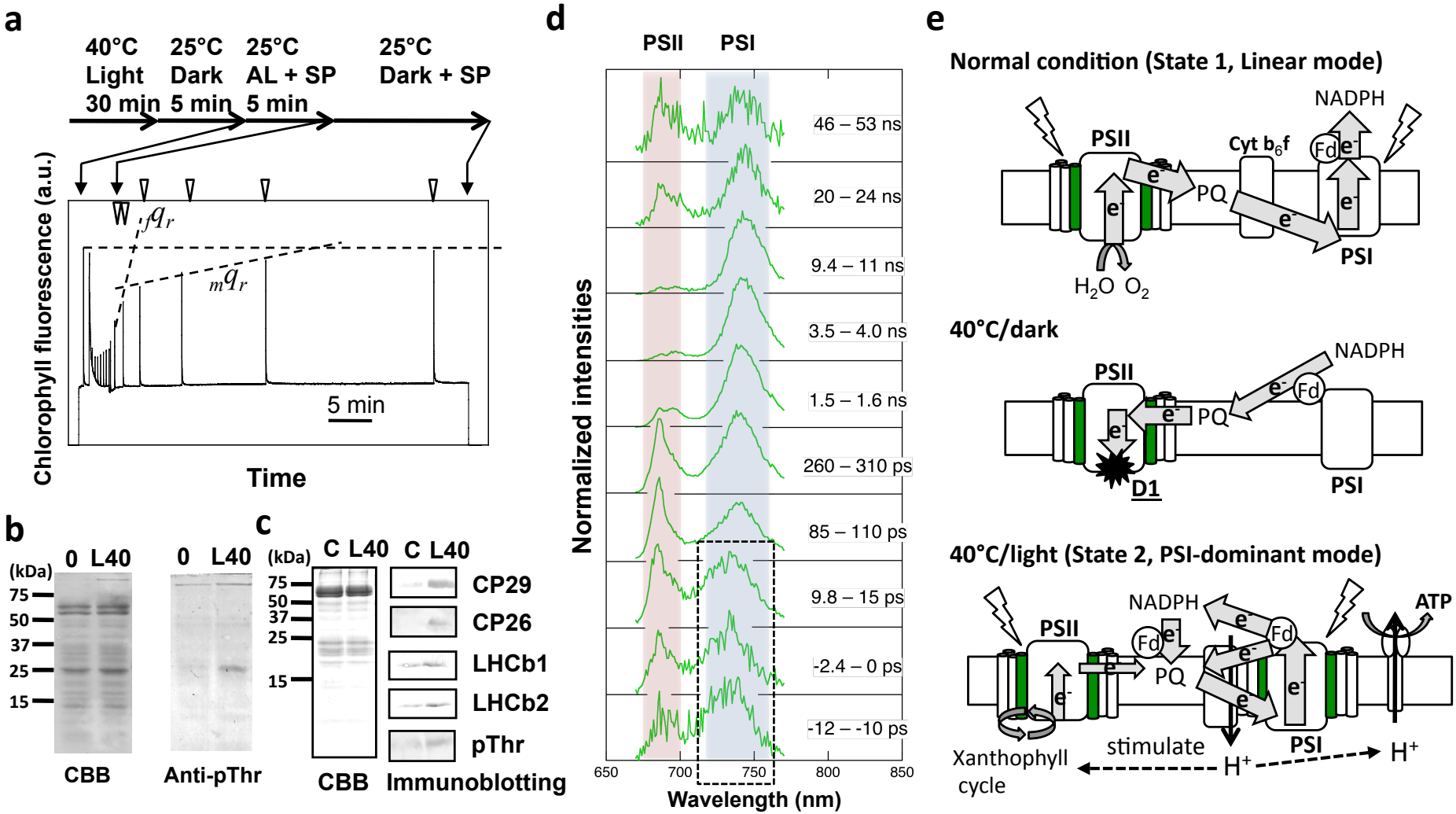
a
40°C Light 30 min
25°C Dark 5 min
25°C AL + SP 5 min
25°C Dark + SP
Chlorophyll fluorescence (a.u.)
$_fq_r$
$_mq_r$
5 min
Time
b
0 L40
(kDa)
75
50
37
25
15
CBB
0 L40
Anti-pThr
c
C L40 C L40
(kDa)
75
50
37
25
15
CP29
CP26
LHCb1
LHCb2
pThr
CBB
Immunoblotting
d
PSII PSI
46 – 53 ns
20 – 24 ns
9.4 – 11 ns
3.5 – 4.0 ns
1.5 – 1.6 ns
260 – 310 ps
85 – 110 ps
9.8 – 15 ps
-2.4 – 0 ps
-12 – -10 ps
Normalized intensities
650 700 750 800 850
Wavelength (nm)
e
Normal condition (State 1, Linear mode)
NADPH
PSII
Cyt b_6f
Fd
PQ
PSI
H_2O O_2
40°C/dark
NADPH
PSII
PQ
D1
PSI
40°C/light (State 2, PSI-dominant mode)
NADPH
ATP
PSII
PQ
PSI
Xanthophyll cycle
stimulate
H^+
H^+

Fig. 4