学位論文

Responses of the photosynthetic electron transport system to fluctuating light

（変動光に対する光合成電子伝達系の応答）

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ABSTRACT

Light energy absorbed by chloroplasts drives photosynthesis. When absorbed light is in excess, the thermal dissipation systems of excess energy are induced and the photosynthetic electron flow is regulated, both contributing to suppression of reactive oxygen species production and photodamages. Various regulation mechanisms of the photosynthetic electron flow and energy dissipation systems have been revealed. However, most of such knowledge has been obtained by the experiments conducted under controlled conditions with constant light, whereas natural light condition is drastically fluctuated. To understand photosynthesis in nature, we need to clarify not only the mechanisms that raise photosynthetic efficiency but those for photoprotection in fluctuating light. Although these mechanisms appear to be well balanced, regulatory mechanisms achieving the balance are little understood.

To assess roles of the cyclic electron flow around PSI (CEF-PSI) and O_2-dependent alternative pathways including the water-water cycle in fluctuating light (FL), I grew the wild type and *pgr5* mutant of *Arabidopsis thaliana* in continuous light for 8 h per day, and measured chlorophyll fluorescence and P700 absorbance changes in their leaves in the FL alternating between 240 (HL) and 30 μmol photon m^{-2} s^{-1} (LL) every 2 min. At 20% O_2, the photochemical quantum yield of PSII decreased, in particular in *pgr5*, soon after the start of the fluctuating light treatment. PSI of the *pgr5*

plants was markedly photoinhibited by this treatment for 42 min. Slight PSI photoinhibition was also observed in the wild type. I measured energy sharing between PSII and PSI and estimated the electron transport rates through PSII, ETR(II), and through PSI, ETR(I). *pgr5* showed larger energy allocation to PSI. In contrast to the wild type, the ratios of ETR(I) to ETR(II) in the *pgr5* plants were high in LL but lowered in HL at 20% O_2 due to the acceptor-side limitation on PSI. At 2.7 or 0% O_2, the CEF-PSI of the *pgr5* plants was enhanced, the acceptor-side limitation of PSI was released, and PSI photoinhibition was not observed. The results suggest that the light fluctuation is a potent stress to PSI and that the CEF-PSI is essential to protect PSI from this stress.

To assess the effects of short-term fluctuating light on photoinhibition of both PSII and PSI, and on regulation of the photosynthetic electron transport system, I measured chlorophyll fluorescence and P700 parameters of *A. thaliana* grown in the continuous light in three FLs alternating between the HL for 2 min and LL for 2min, the FL-240/30 (HL at 240 and LL at 30 μmol photons m^{-2} s^{-1}), FL-1200/30 (HL at 1200 and LL at 30 μmol photons m^{-2} s^{-1}) and FL-1200/240 (HL at 1200 and LL at 240 μmol photons m^{-2} s^{-1}). All of the FL caused PSI photoinhibition, but the degree of PSI photoinhibition was similar during three FL treatments. In response to the FL-1200/30, ETR(II) and ETR(I) kept pace with the changes in light intensity. In these FLs, photoprotective systems, such as the energy dissipation in the PSII antenna system and the down-regulation of electron flow by the photosynthetic control

at the cytochrome b_6/f complex, functioned to regulate the linear electron flow. However, the activities of these systems were insufficient in the FL-240/30. Thus, ETR(II) and ETR(I) in HL phases in the FL-240/30 decreased stepwise with the cycle. These results suggest that differences in modes of light fluctuation have distinct effects on regulation of the photosynthetic electron transport system. I examined the roles of photosynthetic alternative electron flows in response to the FL. The over-expression line of PGR5 showed the marked tolerance to the FL. In addition, continuous measurements of the changes in the electrochromic pigment shift showed that the rate of H^+ effluxes via the H^+-ATPase in chloroplasts did not decrease with the cycles. This may explain why PSI photoinhibition did not enhance PSII photoinhibition in the FL. I suggest that the alternative electron flows, especially the PGR5-mediated cyclic electron flow around PSI, contribute significantly to the compensation of electron flow through PSI, and consequently keep the whole electron transport safely.

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Abbreviations

ΔA_{max}	maximum level of P700 signal in the dark
ATP	adenosine triphosphate
CEF	cyclic electron flow
CEF-PSI	cyclic electron flow around PSI
Chl	chlorophyll
CL	continuous light
CL-240	continuous light at 240 μmol photons m^{-2} s^{-1}
CL-1200	continuous light at 1200 μmol photons m^{-2} s^{-1}
Cyt *b6/f*	cytochrome *b6/f*
DIRK	dark-interval relaxation kinetics
ECS	electrochromic pigment absorbance shift
ETR	electron transport rate
ETR(I)	electron transport rate through PSI
ETR(II)	electron transport rate through PSII
Excess energy	rate of excess energy production
F_0	minimal fluorescence
f_{PSII}	share of absorbed light energy allocated to PSII
FL	fluctuating light
FL-240/30	fluctuating light that altered between HL phase at 240 μmol photons m^{-2} s^{-1} for 2 min and LL phase at 30 μmol photons m^{-2} s^{-1} for 2 min
FL-1200/30	fluctuating light that altered between HL phase at 1200 μmol photons m^{-2} s^{-1} for 2 min and LL phase at 30 μmol photons m^{-2} s^{-1} for 2 min
FL-1200/240	fluctuating light that altered between HL phase at 1200 μmol photons m^{-2} s^{-1} for 2 min and LL phase at 240 μmol photons m^{-2} s^{-1} for 2 min
flg22	peptide derived from bacterial flagellin
FLP	fluctuating light photoinhibition

F_m	maximal fluorescence
FQR	ferredoxin-plastoquinone reductase
F_s'	steady-state fluorescence in light
F_v/F_m	maximum quantum yield of PSII
H^+	proton
H^+-ATPase	H^+-exporting ATP synthase
HL	high light
I_A	absorbed PPFD
k_{pi}	rate of photodamage
$k_{pi,PSII}$	rate of photodamage of PSII
$k_{rec.PSII}$	rate of PSII recovery from photodamage
LED	light-emitting diode
LEF	linear electron flow
LHCII	light harvesting chlorophyll-protein complex II
LL	low light
MAP	Mehler-ascorbate peroxidase
NDH	NADH dehydrogenase-like complex
NDH-mediated CEF	NDH-mediated cyclic electron flow around PSI
NPQ	non-photochemical quenching
P515	elechtrochromic pigment absorbance shiftvia dual-wavelength (550-515 nm) transmittance changes
P700	reaction center of PSI
PAMP	pathogen-associated molecular pattern
PC	plastocyanin
PGR5	PROTON GRADIENT REGULATION5
PGR5-mediated CEF	PGR5-mediated cyclic electron flow around PSI
ΔpH	proton gradient
ΔΨ	thylakoid membrane potential
pmf	proton motive force
PPFD	photosynthetically photon flux density
PQ	plastoquinone

PSI	photosystem I
PSII	photosystem II
PsbS	a specific PSII protein
PTOX	plastid terminal oxidase
q_0	coefficient of the increment of heat dissipation in the light
qE	energy-dependent quenching
qI	photoinhibitory quenching
qL	fraction of open PSII reaction centers
qN	coefficient of non-photochemical quenching
qP	coefficient of photochemical quenching
qT	state transition quenching
RC	reaction center
ROS	reactive oxygen species
WT	wild type
WWC	water-water cycle
Y(I)	photochemical quantum yield of PSI
Y(II)	photochemical quantum yield of PSII
Y(EX)	fraction of energy that is neither used for PSII photochemistry nor dissipated as heat in PSII
Y(NA)	non-photochemical quantum yield due to the acceptor-side limitation of PSI
Y(ND)	non-photochemical quantum yield due to the donor-side limitation of PSI
Y(NO)	quantum yield of non-regulated energy dissipation at PSII centers
Y(NPQ)	quantum yield of regulated energy dissipation at PSII centers

CHAPTER 1.

General introduction

Light is the ultimate resource for photosynthesis, and its intensity (irradiance) drastically changes with time in nature. Many factors including seasons, daily solar movement, cloud cover and canopy architecture combine to produce complex patterns of changes in irradiance in time and space (Pearcy 1990). Plants, therefore, experience dynamic fluctuations of irradiance even when they are in open habitats. Understory plants experience more frequent, short-term irradiance fluctuations due to the leaves and stems of other plants above them (Pearcy 1983; Pearcy 1990; Chazdon 1988; Pearcy et al. 1994; Vierling and Wessman 2000). Plants have to cope with such light fluctuations of various time scales employing several mechanisms (Grieco et al. 2012; Rochaix et al. 2012; Suorsa et al. 2012). These include mechanisms increasing efficiency of photosynthesis as well as those increasing efficiencies of photoprotection (Alter et al. 2012). The balances of these mechanisms should be of supreme importance for actual plant life in nature.

Efforts have been made to clarify how the photosynthetic machinery responds to short-term changes in irradiance. Our knowledge of the dynamics of photosynthesis has been advanced by these studies (Pearcy 1990; Kirschbaum et al. 1998; Külheim et al. 2002; Alter et al. 2012; Suorsa et al. 2012). However, the complex interactions between the fluctuating light

and the dynamics of photosynthesis have not been fully clarified yet. This chapter focuses on recent experimental approaches that have advanced our understanding of 'effects of fluctuating light on long-term response (acclimation) of electron transport system' and 'effects of fluctuating light on short-term response of the electron transport system'.

1.1. Controls of photosynthetic electron flow in fluctuating light

Plants are able to acclimatize their photosynthetic characteristics to their growth light environments. The acclimation of the photosynthetic apparatus, such as changes in the amount of antenna proteins, PSII/PSI stoichiometry, and the contents of electron transport components and enzymes (Anderson et al. 1995), requires several hours to a week. In the longer period encompassing many generations, adaptation occurs, which involves genetic changes leading to adjustments to the light environments although there is an exception. The photosynthetic characteristics brought about by acclimation and/or adaptation to growth irradiance levels were studied intensively (Chazdon et al. 1996; Le Roux et al. 2001; Rothstein and Zak 2001; Oguchi et al. 2005; Oguchi et al. 2006). In these studies, however, dynamic fluctuations of light environments were not paid much attention. In daytime, plants are exposed to changes in irradiance on various timescales, typically in the order of seconds to minutes or hours, but sometimes even milliseconds. Plants have to

cope with such changes in irradiance by various mechanisms that regulate light-harvesting capacity (for example, NPQ; non-photochemical quenching), electron flows, and enzymatic activities (Fig. 1).

The term 'sunfleck' has been used frequently in the literature to describe strong light pulses in natural environments. The duration and distribution of sunflecks are highly variable, and such sunflecks exert substantial effects on CO_2 assimilation and growth of plants (Kirschbaum and Pearcy 1988). The sunfleck light regime may be separated into the periods of multiple sunflecks and the periods with few or no sunflecks (Vierling and Wessman 2000). Leaves in the understory may receive only a few sunflecks or up to 300 or more sunflecks per day. Most of these sunflecks are shorter than 10 s (Pearcy 1983; Chazdon 1988). In the understory of a tropical rain forest, sunflecks longer than 120 s represent only 5% in number but contribute more than 75% of the total daily photosynthetic active photon flux density (PPFD) (Pearcy et al. 1994). In a deciduous forest in early spring, when tree branches have no leaves, irradiance on the forest floor changes more dynamically and contribution of longer sunflecks was greater compared with the situation in summer (Kono, personal observation).

In the 1980s, Pearcy and co-workers revealed that understory plants utilize sunflecks efficiently. They demonstrated that retention of the proton gradient (ΔpH) across the thylakoid membrane and the metabolites of Calvin-Benson cycle are essential for efficient post-illumination CO_2 assimilation (Pearcy 1990; Kirschbaum et al. 1998). In the high-light periods of the fluctuating light in the order of minutes, the electron transport system

is over-reduced and NPQ is developed. The qE-quenching dissipates excess light energy during the high-light periods. In the low-light periods, the qE-quenching can be inactivated within minutes to allow maximum photosynthetic electron transport (Demmig-Adams and Adams 1992; Demmig-Adams et al. 1996; Horton et al. 1996). Porcar-Castell et al. (2006) constructed a dynamic model of PSII quantum yield taking account of the adjustments of the NPQ processes, since steady-state models cannot describe dynamics of the photosynthetic electron flow in fluctuating light. The results showed that both the changes in irradiance on the timescales of seconds to minutes and those in the activation state of Calvin-Benson cycle enzymes influenced the partitioning of energy between NPQ and the photosynthetic electron flow. Although this approach is attractive, we need to prove the model prediction experimentally. The slow relaxation of NPQ after a high-light period would transiently limit CO_2 assimilation in the subsequent low-light period. The high activity of CEF-PSI would support the post-illumination CO_2 assimilation at a high rate via providing ATP and thereby alleviate this problem.

To understand photosynthetic responses of plants to fluctuating light we need to evaluate the responses to the consecutive sunflecks, which are observed in natural environments. Photosynthetic responses to the consecutive sunflecks of alternating low- and high-light are distinct from those to the continuous low- and high-light (Fietz and Nicklisch 2002; Hjelm and Ogren 2004; Nedbal et al. 2005; Porcar-Castell et al. 2006; Wagner et al. 2006). In other words, responses to fluctuating light could not be understood

by our knowledge of acclimation to continuous low- or high-light only (Fietz and Nicklisch 2002).

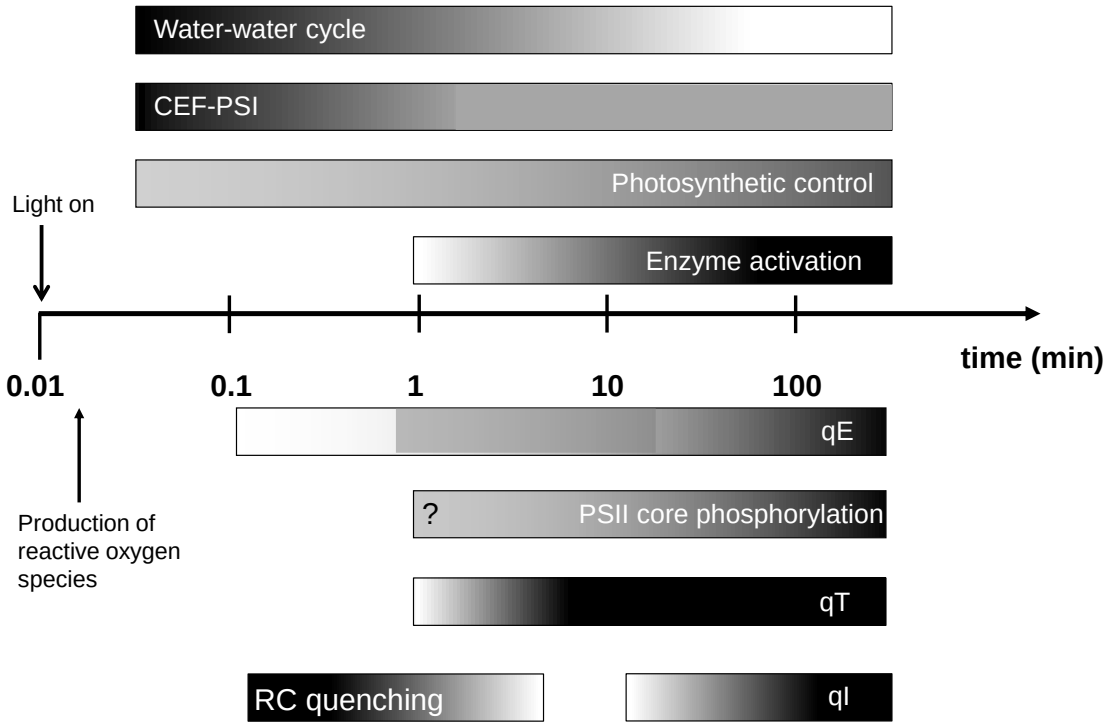


Figure 1. Major mechanisms that occur in response to irradiance in plants. The water-water cycle, Mehler-ascorbate peroxidase pathway (Asada cycle); CEF-PSI, cyclic electron flows around PSI; Photosynthetic control, control of the electron flow by the Cyt b_6/f complex; Enzyme activation, activation of key enzymes in the Calvin-Benson cycle by thioredoxin; qE, qE-quenching; PSII core phosphorylation, photoprotection by phosphorylation of PSII core proteins; qT, qT-quenching; RC quenching, quenching within active PSII reaction center (reaction center quenching); and qI, qI-quenching. ? in PSII core phosphorylation denotes ambiguity of the initiation time scale. Based on a diagram proposed by Eberhard et al. (2008) with modifications. The timescales adopted here are based on the data from various sources mostly cited in the text, but should not be regarded as solid values, because the timescales and the extents of contributions of these regulation mechanisms

differ depending on the experimental conditions.

1.2. Sun and shade-type chloroplasts

Differences between the sun- and shade-type chloroplasts have been well documented (Lichtenthaler 1981; Lichtenthaler et al. 1981; Lichtenthaler et al. 1982; Lichtenthaler 1984; Hjelm and Ogren 2004; Nedbal et al. 2005; Wagner et al. 2006). In shade-type chloroplasts, the content of the light harvesting complexes relative to core complexes is higher than in sun-type chloroplasts (Anderson 1986; Evans 1989; Hikosaka and Terashima 1995). In low light, it is more economical to increase light capture by the light harvesting complexes rather than to have core complexes (Hikosaka and Terashima 1995). The thylakoid membrane structure also differs. Shade-type chloroplasts typically show higher density of thylakoids per chloroplast sectional area, more extensive grana stacks, and thereby more granal thylakoids than sun-type chloroplasts. These features should influence the capacity of the photosynthetic electron transport.

Thylakoid membrane protein complexes distribute heterogeneously between the granal and stroma-exposed regions of the thylakoids. The photosystem II (PSII) complexes are mostly located in grana stacks, whereas photosystem I (PSI) and the H^+-ATP synthase are mostly located in the stroma-exposed thylakoids. The thylakoid architecture and protein distribution dynamically change according to light intensity (Rozak et al. 2002; Kirchhoff et al. 2011; Anderson et al. 2012; Herbstová et al. 2012; Kirchhoff 2013). A recent study, using spinach leaves grown in continuous

light, and kept in the dark, has revealed that most of the PSII subunits distributed in the grana thylakoids and grana margins rather equally, but low molecular mass subunits including the PsbS protein were found in the grana thylakoids (Suorsa et al. 2013). Intriguingly, PROTON GRADIENT REGULATION5 (PGR5) was distributed evenly between granal and stroma-exposed thylakoids, whereas PGR5-LIKE PHOTOSYNTHETIC PHENOTYPE1 (PGRL1) was enriched in stroma-exposed thylakoids.

1.3. Non-photochemical quenching

1.3.1. Energy-dependent quenching

Non-photochemical quenching (NPQ) contributes to down-regulation of the photosynthetic electron transport in the chloroplast. NPQ of excess excitation plays a protective role, which prevents over-acidification of the lumen and decreases the damage to PSII in high irradiance. Various mechanisms contribute to NPQ, and there are three components on the basis of the time constants of the NPQ relaxation kinetics in the dark following a period of illumination: (i) qE, energy-dependent quenching, requires acidification of the thylakoid lumen and is relaxed within seconds to minutes; (ii) qT, state transition quenching, is caused by the changes in the relative size of the antennae associated with PSII and PSI; (iii) qI, photoinhibitory quenching, is

caused by photoinhibition of PSII and shows very slow relaxation (repair) kinetics in the range of hours.

The down-regulation of PSII activity by qE-quenching is reversible and flexible (Bianchi et al. 2010; Ruban et al. 2012), which is triggered by the light-induced acidification of the thylakoid lumen. Acidification of the lumen induces the NPQ through protonation of a specific PSII protein (PsbS) and activation of the xanthophyll cycle. PsbS, an integral membrane protein that does not appear to bind pigments (Bonente et al. 2008) functions as a sensor of lumen pH (Li et al. 2000; Niyogi 2000; Li et al. 2002; Li et al. 2004). Although the biochemical mechanism is not yet understood, protonation of PsbS seems to promote a rearrangement of the PSII supercomplex in grana. The rearrangement is necessary for rapid induction of NPQ (Betterle et al. 2009; Goral et al. 2012). Activation of the xanthophyll cycle is achieved through activation of violaxanthin de-epoxidase by acidic pH and results in de-epoxidation of violaxanthin to zeaxanthin (Demmig-Adams 1990; Demmig-Adams et al. 2012). The deepoxidation may induce conformational changes of PSII to a quenching mode. Alternative explanation is that zeaxanthin quenches excited state of chlorophyll and eventually dissipates as heat (Blankenship 2001). Collaboration of these two pH-induced processes, PsbS protonation and activation of xanthophyll cycle, allows the accomplishment of a maximal performance of NPQ (Muller et al. 2001; Ruban et al. 2012). According to a photodamage hypothesis claiming that photoinhibition is induced by excess light energy, namely the light energy reaching closed PSII reaction centers, these pH-induced processes contribute

to suppression of photoinhibition of PSII (Niyogi 2000; Vass 2011).

1.3.2. qE-quenching in fluctuating light

Physiological importance of qE-quenching for plant performance is suggested by the observation using *A. thaliana* mutants, *npq1* and *npq4*, with an impaired xanthophyll cycle and deficient in the PsbS protein, respectively. Fitness components, such as the seed number per plant, of these mutants were significantly reduced when grown outdoors under natural fluctuating light conditions or in an artificially fluctuating light in a growth chamber. However, when grown in constant light, these mutants showed no phenotypic defects. These demonstrate that dynamically regulated non-photochemical energy control is an important mechanism providing a strong fitness advantage under field conditions (Külheim et al. 2002; Kulheim and Jansson 2005). Field-grown mutants were photo-inactivated to a greater degree than wild type, whereas the mutant plants grown in the continuous light in the growth chamber showed no photoinhibition of PSII. These results demonstrate that qE-quenching confers an advantage to the wild-type plants through increasing the dynamic range of photosynthesis and thereby allowing the photosynthetic apparatus to utilize light energy optimally. Although it is generally believed that NPQ plays a role in photoprotection (Demmig-Adams and Adams 1992; Horton et al. 1996; Niyogi 1999), NPQ indirectly protects the repair process of photodamaged PSII from the oxidative stress by suppression of production of the reactive oxygen species. It is also worth noting that the direct role of qE-quenching in photoprotection has been

questioned in the two-step hypothesis of PSII photoinhibition (for the two-step hypothesis, see Sarvikas et al. 2006). NPQ may also contribute to the well-balanced excitation of the two photosystems (Peterson and Havir 2001).

Recently, interaction between the regulation of NPQ and plant-pathogen has been suggested. Plants sense potential pathogens by recognizing the conserved pathogen-associated molecular patterns (PAMPs). Gohre et al. (2012) demonstrated that a long-term treatment of *A. thaliana* plants with flg22, a peptide derived from bacterial flagellin, one of the PAMPs, triggered the increase in NPQ in chloroplasts and promoted expression of defense-related genes, but a receptor mutant, *flg22,* did not (Gohre et al. 2012). They have proposed that regulation of NPQ was an intrinsic component of the plant defense program. It may be important, therefore, to note that the plants grown in the field are exposed to more or less a variety of other biotic stresses, such as herbivory by insects and infection by pathogens. These stresses may explain the decrease in fitness of the *npq4* mutant in the field (Külheim et al. 2002).

1.3.3. Enhanced photoprotection and photosynthetic capacity

Depending on whether the same amount of photons is given as a short, bright 'pulse (sunfleck)' or as a longer continuous light, acclimation processes are different. Alter et al. (2012) demonstrated acclimation of *A. thaliana* to fluctuating light regimes of different duration, frequency, and/or intensity but

of the same total PPFD per day. Wild-type plants grown in a fluctuating light regime with short- (less than 12 min) and high- (above 650 μmol photons m^{-2} s^{-1}) lightflecks showed enhanced activities of photoprotection and energy dissipation, presumably because they were unable to utilize efficiently the strong light energy provided in this manner. These acclimation mechanisms involved reorganization of the pigment-protein complexes, resulting in faster light-induced NPQ and the increase in the NPQ capacity, as well as an enhanced activity of superoxide dismutase. Effective acclimation responses to the short lightflecks enabled these plants to cope with photo-oxidative stress induced by these lightflecks. On the other hand, the fluctuating light with longer (for example, 40 min) lightflecks at high light caused plants to up-regulate their electron transport capacity rather than NPQ.

1.3.4. Reaction center quenching

In addition to the dissipation of excess light energy occurring in the PSII antenna via the PsbS protonation and xanthophyll cycle, there is another ΔpH-dependent NPQ process within the PSII reaction center, sometimes referred to as 'reaction center quenching' (Weis and Berry 1987; Krause 1988; Krause and Weis 1991; Walters and Horton 1993). It has been proposed that the conversion of photochemically active, fluorescent, closed PSII reaction centers into photochemically inactive, non-fluorescent PSII reaction centers may serve as an effective mechanism for energy dissipation. The proportion

of the inactive PSII centers to the active centers is dependent on the ΔpH across the thylakoid membrane and the proportion of closed reaction centers measured as the relative reduction state of Q_A (Weis and Berry 1987; Krause and Weis 1991). Thus, over-reduction of Q_A (increase in E_m of Q_A/Q_A^-) has been suggested to be a major prerequisite for this quenching within the PSII reaction center (Krause 1988; Horton 1993; Bukhov et al. 2001; Öquist and Huner 2003). This reversible interconversion of PSII from the photochemical energy transducer to non-photochemical energy quencher could protect the photosynthetic apparatus from the environmental stresses, such as low temperature and high light (Ivanov et al. 2006), which potentially induce the high excitation pressure. Non-radiative charge recombination between Q_A^- and the donor side of PSII has been suggested as a mechanism for dissipating excess energy within the PSII reaction center (Ivanov et al. 2008). This quenching was shown to be extensive during the first several seconds of illumination of dark-adapted plants, even in low light, indicating that the reaction center quenching is triggered by the transient over-acidification of the thylakoid lumen. On the other hand, its disappearance would result from the relaxation of ΔpH across the thylakoid membrane and the activation of the Calvin-Benson cycle (Finazzi et al. 2004). This reaction center quenching would commonly occur in response to sudden increases in the irradiance, depending on the balance between the rate of electron flow and that of the Calvin-Benson cycle. Thus, we propose that the reaction center quenching may serve as an effective response to fluctuating light, especially when periods of extremely low light are long enough to inactivate the Calvin-

Benson cycle enzymes and convert zeaxanthin to violaxanthin.

In contrast to the reversible quenching within the PSII reaction center, the possible involvement of irreversibly photoinhibited PSII as a quencher has been also proposed. Importance of the irreversibly photoinactivated centers as the quencher, would increase with the severity of photoinhibition. This was suggested by the fact that the decline of the proportion of the active PSII did not follow the first-order kinetics (Lee et al. 2001; Chow et al. 2002). Further studies demonstrated that these damaged PSII complexes function as strong quenchers of excess light energy, thus effectively protecting the remaining active PSII reaction centers from photodamage (Matsubara and Chow 2004). However, recent studies (Sarvikas et al. 2010; Kou et al. 2012) have raised a question about the quenching by the photoinhibited PSII because, in these studies, photoinhibition of PSII followed the first-order kinetics (Tyystjärvi et al. 1994; Tyystjärvi and Aro 1996). These discrepancies should be solved. Beside the discrepancy concerning the quenching by the photoinhibited PSII, PSII photoinhibition protects photodamage of PSI, because electron flow to PSI via PSII decreases and production of reactive oxygen species is also suppressed (Sonoike 1996). Although we do not detail in this chapter, PSII photoihibition-repair cycle would be important process that indirectly regulates the photosynthetic electron flows, suppresses formation of reactive oxygen species, and protects PSI from photodamage (Sonoike 1996; Takahashi and Murata 2008; Tikkanen et al. 2013).

1.4 PSI in fluctuating light

1.4.1 Photosynthetic control

"Photosynthetic control" at the Cyt b_6f complex also down-regulate the electron transport (West and Wiskich 1968; Rumberg and Siggel 1969; Hall et al. 1971). This "photosynthetic control" works depending on the phosphate potential, [ATP]/([ADP] x [Pi]), where [ATP], [ADP] and [Pi] stand for concentrations of ATP, ADP and Pi, respectively (for reviews, see Kramer et al. 1999 and Tikhonov 2013). When ATP synthesis occurs intensively, protons are excreted from the lumen to stroma through the H^+-ATP synthase, preventing excessive acidification of the lumen. The moderately acidic pH allows high rate of electron transfer to PSI. However, when ADP and Pi are in shortage, production of ATP is suppressed, lumen pH decreases and thereby the electron transport is decelerated (Takizawa et al. 2008; Kiirats et al. 2009). This acidification of the lumen affects PSII and the Cyt b_6f complex (Takizawa et al. 2007). The pH-dependent modulation of PSII may be accomplished by decelerating the protolytic steps of PSII (Tikhonov et al. 1981). In the proton-coupled electron transport events in the Cyt b_6f complex, the oxidation of plastoquinol (PQH_2) at the Q_0 site is the rate-limiting step. A recent study using *Nicotiana tabacum* indicates that the H^+-ATPase also contributes to the photosynthetic control (Rott et al. 2011). Thus, the light-induced acidification of the lumen is the main factor of the feedback control

of the linear electron transport in chloroplasts. This photosynthetic control may function as photoprotective mechanism in fluctuating light, especially in relatively-prolonged sunflecks in combination with ΔpH-dependent NPQ. However, photoprotective mechanism due to the photosynthetic control is too slow to be active enough upon the extremely rapid increase in light intensity, since the light-induced acidification of the lumen has a lag.

1.4.2. PSI photoinhibition

How is PSI affected under fluctuating light? In contrast to PSII that is highly susceptible to photodamage, it was widely believed that PSI is efficiently protected against photodamage. While PSI is quite resistant to typical high light stress, it is very sensitive to photodamage under certain conditions such as chilling temperatures in certain plants. In *Cucumis sativus*, a chilling sensitive plant, chilling of leaves at moderate light irradiance gives damage to PSI with little damage to PSII (Terashima et al. 1994; Sonoike et al. 1995; Sonoike et al. 1997; Sonoike 2011). Photodamage to PSI has been also shown in other plants (Havaux and Davaud 1994; Ivanov et al. 1998; Tjus et al. 1999). Photoinhibition of PSI to an extent similar to that of PSII photoinhibition has been reported in chilling-tolerant plants such as *A. thaliana* (Zhang and Scheller 2004). PSI photoinhibition would stimulate PSII photodamage because electron flow from PSII is disrupted and electron transport chain is over-reduced, whereas PSII photoinhibition protects PSI from photodamage

(Sonoike 1996; Tikkanen et al. 2013).

1.4.3. The role of PGR5 protein in PSI protection in fluctuating growth light

A recent pioneering paper has proposed an important role of PGR5 in protection of PSI under the fluctuating growth light in *A. thaliana* (Suorsa et al. 2012). The *pgr5* mutant, isolated on the basis of its high chlorophyll fluorescence at high irradiance (Munekage et al. 2002), showed no growth when grown in the drastically fluctuating light, alternating low light for 5 min and high light for 1 min (Tikkanen et al. 2010; Suorsa et al. 2012). In more moderately fluctuating growth light, the plants grew to some extent but the PSI complex in this mutant was photodamaged. They also reported that, under the constant growth light, the *pgr5* mutant did not show any visible growth phenotype irrespective of the growth irradiance levels. The *pgr5* mutant is deficient in development of ΔpH across the thylakoid membrane with the increase in irradiance (Munekage et al. 2002), but under constant light conditions *pgr5* forms a normal ΔpH, in which ATP production is probably driven in a rate similar to that in wild type (Suorsa et al. 2012). In the absence of PGR5, not only rapid induction of qE-quenching but also the pH-dependent photosynthetic control of linear electron flow via the Cyt b_6f complex is suppressed. Therefore, the combination of suppression of NPQ and uncontrolled linear electron flow readily leads to an endangered state for PSI, as demonstrated in the *pgr5* under fluctuating growth light conditions (Suorsa et al. 2012). Inability of the *pgr5* to slow down electron flow via the Cyt b_6f complex upon increase in irradiance results in over-reduction of the

acceptor-side of PSI.

The photoprotection of PSI by PGR5 (Suorsa et al. 2012) and the excitation energy balance provided by the steady-state LHCII phosphorylation (Pesaresi et al. 2009; Tikkanen et al. 2010; Grieco et al. 2012) in fluctuating growth light are key factors for acclimation in that both of these contribute to maintenance of the activity of PSI but not PSII (Grieco et al. 2012). According to Suorsa et al. (2012), when plants were grown under the fluctuating light condition with alternating 5 min of low light (50 μmol photons m^{-2} s^{-1}) and 1 min of high light (500 μmol photons m^{-2} s^{-1}) during the photoperiod, the lack of PGR5 in the *pgr5* was not compensated for by up-regulation of the NDH-mediated CEF. The complete absence of the NDH complex (*ndho* mutant) did not cause growth suppression in the fluctuating growth light. Likewise, PTOX did not seem to play a crucial role under the fluctuating growth light. Although the over-expression of antioxidant enzymes such as superoxide dismutase and ascorbate peroxidase in the *pgr5* plants decreased the level of reactive oxygen species, PSI was not protected against fluctuating light-induced stress. According to Grieco et al. (2012), the functionality of PSI and the response to fluctuating light are regulated by PGR5-dependent control of electron flow in cooperation with steady-state LHCII phosphorylation and NPQ-dependent electron flow control. In short-term response, the balanced excitation and redox balance provided by the steady-state phosphorylation of LHCII is essential to maintain PSI.

1.5 Aims of the study

In my studies for the doctoral thesis, I have done simultaneous measurements of chlorophyll fluorescence and P700 absorption changes at 830 nm with leaves of *Arabidopsis thaliana* plants.

In the experiments described in chapter 2, I have used *pgr5* mutant and measured PSII and PSI parameters at 20, 2.7 and 0% O_2 concentrations to assess the roles of the cyclic electron flow around PSI and O_2-dependent alternative pathway including the water-water cycle in response to short-term fluctuating light.

In the study for chapter 3, I have done photionhibition experiments to examine the effects of some modes of fluctuating light on the H^+-ATPase and especially on photoinhibition of two photosystems in chloroplasts. Further, I have measured the responses of PSII and PSI parameters to short-term fluctuating light to evaluate the effects of fluctuating light for 160 min on regulation of the photosynthetic electron transport system.

In chapter 4, I discuss the results of these results and propose several further studies.

CHAPTER 2

Roles of the cyclic electron flow around PSI (CEF-PSI) and O_2-dependent alternative pathways in regulation of the photosynthetic electron flow in short-term fluctuating light in *Arabidopsis thaliana*

2.1. Introduction

Even in open habitats, plants experience dynamic fluctuations of light because of clouds. Understory plants experience more frequent, short-term light fluctuations due to leaves and stems of other plants above them in addition to clouds. Plants have to cope with these light fluctuations of various time ranges. In constant low light, plants can use most of light energy in driving photochemistry. In contrast, in constant high light, energy transfer from antenna chlorophylls to the photosystem II (PSII) reaction center is suppressed and the excess energy is dissipated as heat. This process prevents photoinhibition. When the light intensity fluctuates between low and high levels very rapidly, however, it is not possible for chloroplasts to synchronously switch on and off the heat dissipation system with the light fluctuation because both induction and deactivation of the heat dissipation system require at least several minutes (Muller et al. 2001). Plants must have more rapid systems to cope with very rapid light fluctuations.

Photosynthetic electron transport primarily occurs via a linear pathway, in which electrons flow from water via PSII and cytochrome b_6f complex to PSI and reduce $NADP^+$ to NADPH. The linear electron flow (LEF) generates the transmembrane electrochemical potential difference of H^+, through water splitting by PSII in the thylakoid lumen and translocation of H^+ across the thylakoid membrane by the Q cycle. The electrochemical potential difference thus produced drives the H^+-ATP synthase to produce ATP. Low pH in the thylakoid lumen causes de-epoxidation of violaxanthin to

zeaxanthin via antheraxanthin and protonation of the PsbS protein, both of which contribute to the heat dissipation, which can be measured fluorometrically as the non-photochemical quenching (NPQ).

In addition to the LEF system, there are two PSI cyclic electron flow (CEF) systems (Shikanai 2007): the NADH dehydrogenase-like complex-dependent pathway (NDH-CEF, Burrows et al. 1998; Shikanai et al. 1998; Peng et al. 2011; Yamamoto et al. 2011) and the ferredoxin-plastoquinone reductase pathway (FQR-CEF, Munekage et al. 2002, 2004; DalCorso et al. 2008; Hertle et al. 2013). FQR-CEF involves cytochrome *b*$_6$*f* complex, plastocyanin, PSI, ferredoxin (Fd) and ferredoxin-plastoquinone reductase (FQR). PGR5 was identified as an essential component of the FQR-CEF (Munekage et al. 2002, see below). Very recently, PGRL1 has been proposed to be the elusive FQR (Hertle et al. 2013). In C_4 plants, the cyclic electron flows around PSI (CEF-PSI), particularly the NDH-CEF, operate to supply ATP to the CO_2 concentrating mechanism (Takabayashi et al. 2005) as well as the Calvin Benson cycle. It is noteworthy that the NDH-CEF also involves ferredoxin (Okegawa et al. 2008; Johnson 2011; Yamamoto et al. 2011). It is often claimed that the ATP and NADPH production by the LEF cannot meet the required ATP/NADPH ratio for the photosynthetic carbon fixation by the Calvin-Benson cycle. In particular, when photorespiration occurs at high rates, the required ratio shifts from 3ATP/2NADPH towards 3.5ATP/2NADPH, and thereby shortage of ATP may be more serious (Allen 2002; Shikanai 2007). The CEF-PSI would contribute to producing additional ATP. Another function of CEF-PSI is enhancement of the NPQ, through

generating the electrochemical potential difference of H^+ across the thylakoid membrane (Munekage et al 2002).

The pseudo-cyclic electron flow, also called the water-water cycle (WWC) (Asada 1999) or the Mehler-ascorbate peroxidase (MAP) pathway (Schreiber et al. 1995), is the electron flow from water via PSII, cytochrome *b6/f*, and PSI to molecular oxygen. Since the redox potentials of the electron acceptors on the acceptor side of the PSI complex are sufficiently low to reduce O_2, the electron flow to O_2 occurs, particularly when $NADP^+$ is not available. This results in the formation of reactive oxygen species (ROS), such as O_2^- and H_2O_2 (Asada 1999). Superoxide dismutase and ascorbate peroxidase in the WWC scavenge O_2^- and H_2O_2. NADPH is used to regenerate ascorbate from monodehydroascorbate or dehydroascorbate. Summing up these reactions, electrons are transferred from water to H_2O_2 to form water. Thus, the WWC acts as a large electron sink (Asada 2000). The WWC also generates the electrochemical potential difference of H^+ across the thylakoid membrane, which enhances the non-radiative dissipation of excess light energy observed as the increase in NPQ. Therefore, the WWC is also considered to play roles in dissipation of excess light energy (Osmond and Grace 1995; Osmond et al. 1997; Asada 1999, 2000; Foyer and Noctor 2000; Miyake 2010). The CEF-PSI and WWC are argued to protect plants from damages that occur due to the over-reduction of the thylakoids under stress conditions (Miyake 2010).

An *Arabidopsis thaliana* mutant, *pgr5* (*proton gradient regulation*), was reported to have the impaired electron transfer in FQR-CEF (Munekage et al. 2002). In the screening using the chlorophyll fluorescence imaging

technique, this mutant showed a phenotype similar to that of *npq* mutants (Shikanai et al. 1999). NPQ measurements with this mutant showed an almost complete absence of qE-quenching at high irradiance under steady-state photosynthesis (Munekage et al. 2002). Nandha et al. (2007), however, reported that the capacity of the cyclic electron transport in *pgr5* was comparable to that of the wild-type. They also showed that the electron transport system in *pgr5* was largely reduced under most conditions.

A recent paper has proposed an important role of PGR5 in protection of PSI under the fluctuating growth light in *Arabidopsis thaliana* (Suorsa et al. 2012). The *pgr5* mutant showed no growth when grown in the drastically fluctuating light, alternating low light for 5 min and high light for 1 min (Tikkanen et al. 2010; Suorsa et al. 2012). In a more moderately fluctuating growth light, the PSI complex in this mutant was found to be photodamaged. They also reported that, under the constant growth light, the *pgr5* mutant did not show any visible growth phenotype irrespective of the growth irradiance levels. From these, they argued that the *pgr5* could not maintain redox balance of the electron transfer reactions in the fluctuating light. However, how the redox imbalance occurs in *pgr5* and how the wild-type plants cope with the drastically fluctuating light are still unclear.

The aim of this study was to examine photosynthetic responses of the wild-type (WT) and *pgr5* plants, both grown in the continuous moderate light in the light period, to a fluctuating light using simultaneous chlorophyll fluorescence and P700 measurements under the precise control of gas concentrations. The fluctuating light adopted was alternation of low light for

2 min and high light for 2 min. Even for high light, a moderate level of photosynthetically active photon flux density (PPFD) was chosen. I examined whether photoinhibition of PSI occurred in the mature leaves of the *pgr5* plants in short-term experiments for up to 42 min. Next, I tried to elucidate which of the photosynthetic alternative electron flows was impaired in the *pgr5* plants through examining the effects of O_2 concentrations at various PPFDs.

2.2. Materials and methods

Plant materials

Arabidopsis thaliana wild-type (ecotype Columbia *gl1*) and *pgr5* mutant (Munekage et al. 2002) plants were pot grown in a growth cabinet with white fluorescent light at 90-100 μmol photons m^{-2} s^{-1} for 8-h photoperiod at room temperature of 23℃ and relative humidity of 60%. Plants were irrigated two to three times weekly and were fertilized with Hyponex 6-10-5 solution (Hyponex Japan, Osaka, Japan) diluted to the 1: 1000 strength every irrigation from two weeks after germination. Mature rosette leaves from 7- to 9-week-old plants were used in the experiments. An *A. thaliana* mutant, *crr2-2,* were also used. The growth conditions of these plants were the same as those for the wild type.

Chlorophyll fluorescence and 830 nm absorbance change measurements

Chlorophyll fluorescence and absorption changes at 830 nm were measured simultaneously using a Dual-PAM-100 (chlorophyll fluorescence and P700 absorption analyzer equipped with a P700-dual wavelength-emitter at 830 and 875 nm, Walz, Effeltrich, Germany) with the intact leaf in a hand-made leaf chamber. CO_2 concentration in the leaf chamber was monitored with a LI-6400 (Li-Cor, Lincoln, NE, USA). O_2 concentration in the air was controlled by mixing N_2 gas and O_2 gas using mass flow controllers. Saturation pulses

(SP) from red light-emitting diodes (LEDs; > 8000 μmol photons m^{-2} s^{-1}, 400 ms duration) were applied to determine the maximum chlorophyll fluorescence with closed PSII centers in the dark (F_m) and in the actinic light (F_m'). Maximum photochemical quantum yield of PSII (F_v/F_m) and effective quantum yield of PSII (Y(II)) were calculated as $(F_m - F_0)/F_m$ and $(F_m' - F_s')/F_m'$ (Genty et al. 1989), respectively, where F_s' is the steady-state chlorophyll fluorescence level in the actinic light from red LEDs with wavelength peak at 635 nm, in which chloroplast avoidance movement does not occur and has no effect on assessment of non-photochemical quenching components (Cazzaniga et al. 2013). The coefficient of non-photochemical quenching, qN, was calculated as $(F_m - F_m')/(F_m - F_0')$. F_0' is minimal fluorescence yield in the actinic light and was estimated using the approximation of Oxborough and Baker (1997) as $F_0/(F_v/F_m + F_0/F_m')$. Two other PSII quantum yields, Y(NPQ) and Y(NO) (Genty et al. 1996; Kramer et al. 2004a), which represent the regulated and non-regulated energy dissipation at PSII centers respectively and add up to unity with the photochemical quantum yield (i.e. Y(II) + Y(NPQ) + Y(NO) = 1), were also used. Y(NPQ) and Y(NO) were calculated as $F_s'/F_m' - F_s'/F_m$ and F_s'/F_m, respectively (Hendrickson et al. 2004; Klughammer and Schreiber 2008a). The coefficient of photochemical quenching, qL, a measure of the fraction of open PSII reaction centers, based on the "lake model" of PSII antenna pigment organization, was calculated as $(F_m' - F_s')/(F_m' - F_0')\cdot F_0'/F_s'$ (Kramer et al. 2004a).

In the Dual-PAM-100, $P700^+$ was monitored as the absorption

difference between 830 and 875 nm in a transmission mode. In analogy to chlorophyll fluorescence yield, the quantum yield of PSI was determined using the saturation pulse method (Klughammer and Schreiber 1994; Klughammer and Schreiber 2008b). Maximum level of P700 signal (P700 fully oxidized) in the dark, called P_m, was determined by application of a SP in the presence of far-red light at the wavelength of 720 nm. The zero P700 signal, P_0, was determined when complete reduction of P700 was induced after the SP in the absence of far-red light. P_m' is the maximal P700 signal in the presence of actinic light induced by the SP. The photochemical quantum yield of PSI, Y(I), was calculated from the complementary PSI quantum yields of non-photochemical energy dissipation, Y(ND) and Y(NA), respectively: $Y(I) = 1 - Y(ND) - Y(NA)$. Y(ND) corresponds to the fraction of P700 that is already oxidized by actinic light, and Y(NA) corresponds to the fraction of P700 that cannot be oxidized by a SP to the overall P700. These calculations were made according to Klughammer and Schreiber (2008b). To oxidize the inter-system electron carriers, far-red light was applied from 100 ms before the start of the SP to its cessation. As shown in Fig. 2, Y(I) did not decrease under the constant HL. My preliminary checks showed that the SP of 400 ms duration was enough to induce maximal $P700^+$ oxidation level and to obtain complete reduction level of P700 after the SP. Photodamage by the SP did not occur.

The proportions of the non-photochemical quenching components were determined from the relaxation kinetics of the variable fluorescence (F_v) in the absence of actinic light for 30 min (Quick and Stitt 1989; Walters and

Horton 1991). Relaxation of F_v was monitored with saturation pulses given every 100 s to the leaf. The intervals of 100 s were sufficient to eliminate an effect of saturation pulse on F_v relaxation. The fast-relaxing component of fluorescence quenching was assigned to the energy-dependent mechanism (qE), the intermediate relaxing component was assigned to the state transition (qT), and the slow relaxing component was assigned to the photoinhibitory processes (qI). For quantification of qE, qT and qI, the semi-logarithmic plot of F_v versus time was analyzed considering the relationship $(1 - qN) = (1 - qE) \times (1 - qT) \times (1 - qI)$.

I estimated the electron transport rate through PSI (ETR(I)) and PSII (ETR(II)) simultaneously. In this study, a source of artifacts should be considered for a comparison of ETR(I) with ETR(II). With the blue measuring light, chlorophyll fluorescence signal mainly emitted from the upper layer of the mesophyll cells closest to the emitter detector unit was detected, while the P700 signal detected was more generally from the whole leaf tissue. In high light, these upper cells would be prone to light saturation of photosynthesis and photoinhibition than the cells in the deeper layer (Terashima et al. 2009; Oguchi et al. 2011a and b). To effectively prevent this preferential light saturation of photosynthesis and photoinhibition near the leaf surface, red light at 635 nm of peak wavelength instead of blue light was used as the actinic light. The red actinic light at this wavelength reaches the deeper cell layers, and would cause more even light saturation of photosynthesis and photoinhibition than the blue actinic light.

PSI fluorescence may contribute to total leaf fluorescence (Pfundel

1998; Rappaport et al. 2007). In this study, blue light at 460 nm of peak wavelength was used as the measuring light, except for the data shown in Fig 5, see below. The blue measuring light excites PSII more than that from PSI. In addition, according to Pfundel et al. (2013), emission of PSI fluorescence by the *A. thaliana* leaves is low irrespective of growth PPFD levels.

The P700 signal can be interfered by the absorbance changes of plastocyanin. Up to 10% of the P700 difference absorption signal measured by the DUAL-PAM instrument may be attributable to that of plastocyanin, which shows considerable absorption at both 830 to 870 nm (Kirchhoff et al. 2004). Livingston et al. (2010) compared the results with the Dual-PAM system and those using the two-wavelength deconvolution method (ΔA of 820-950 nm) described by Oja et al. (2004) and concluded that absorbance changes from plastocyanin or other components may not substantially affect the P700 measurements.

Measurements of the share of absorbed light energy allocated to photosystem II

To estimate the share of absorbed light energy allocated to PSI and PSII, simultaneous measurements of O_2 evolution and chlorophyll fluorescence in the leaf were made at 23℃ using a leaf-disk oxygen-electrode system (LD2, Hansatech, Kings Lynn, UK) and a chlorophyll fluorometer (PAM-2500, Walz, Effeltrich, Germany).

Leaf segments were placed in the chamber of the leaf-disk O_2

electrode. When the steady-state rate of O_2 evolution was attained, the quantum yield of PSII photochemistry (Y(II)) was measured. Irradiance of actinic light was increased in a stepwise manner. A Björkman-type lamp equipped with a red color filter of the wavelength centered at 635 nm was used as the light source. The red light was used to mimic the spectrum of the actinic light of the Dual-PAM system. PPFD was altered with neutral density filters (Toshiba, Tokyo, Japan). The air in the chamber contained about 5% CO_2 and 15% O_2. The quantum yield of O_2 evolution ($Y(O_2)$) of the leaf was calculated by dividing the rate of gross O_2 evolution per leaf area (μmol O_2 m^{-2} s^{-1}) by absorbed PPFD. Absorptance of the leaf was measured with a handmade integrating sphere, whose inside was coated with $BaSO_4$, and a quantum sensor (LI-190SA, Li-Cor). When f_{PSII}, the share of absorbed light energy allocated to PSII is less than 0.5, the relationship between quantum yield of O_2 evolution at saturating CO_2 ($Y(O_2)$) and that of PSII electron transport (Y(II)) can be expressed as $Y(O_2) = I_A \times f_{PSII} \times Y(II)/4$, where I_A is the absorbed PPFD (Genty et al. 1989).

This equation, which compares gross O_2 evolution from the whole tissue with Y(II) obtained from the shallow part of mesophyll, may lead to uncertainty in f_{PSII}. The error in f_{PSII} gives rise to uncertainty in ETR(II). Since fluorometrically estimated ETR(II) tends to be underestimated compared with that calculated from the gross O_2 evolution rate, especially at high PPFDs, ETR(I)/ETR(II) ratio calculated with fluorometrical ETR(II) would be overestimated (Kou et al. 2013). To obtain chlorophyll fluorescence signal from the deeper mesophyll cells with the PAM-2500, I used red light

peaked at 630 nm as the measuring light. The use of the red measuring light, rather than blue light, would minimalize the error in estimation of ETR(II), particularly that at relatively low PPFDs. Thus, I used data points obtained at low PPFDs. Effects of fluorescence from PSI would be small at low PPFDs.

Determination of chlorophyll content

Chlorophyll *a* and *b* contents were determined according to Porra et al. (1989).

2.3. Results

Responses of PSII and PSI quantum yields to fluctuating and continuous light

Chlorophyll fluorescence and absorption changes at 830 nm in the intact leaf were measured simultaneously using a Dual-PAM-100 (Walt GmbH, Germany). Changes in the PSII quantum yield, Y(II), in mature leaves of the wild-type (WT) and *pgr5* plants were measured in the light regime that alternated between high light (HL) at 240 μmol photon m^{-2} s^{-1} for 2 min and low light (LL) at 30 μmol photon m^{-2} s^{-1} for 2 min, for a total of 42 min (Fig. 1). The leaf was kept in a small hand-made chamber, and CO_2 and O_2 gas concentrations in the chamber were regulated with mass-flow controllers. Unless otherwise stated, the CO_2 and O_2 concentrations were 390 ppm and 20%, respectively. In the leaves of WT plants grown in the constant light at 90 - 100 μmol photon m^{-2} s^{-1} for 8 h /day, Y(II) at the end of each LL-period decreased with the cycle, but Y(II) at the end of each HL-period did not change after attaining the steady value around 0.45. In *pgr5*, Y(II) in LL-period decreased with the cycle more markedly than in WT. Y(II) in HL-period also decreased after the fifth cycle. In the last cycle, Y(II) in LL-period became 0.4, approaching that in HL-period being around 0.3.

To compare the photosynthetic responses in the fluctuating light and those in the continuous light, I measured changes in Y(II) and the PSI quantum yield, Y(I), in the constant HL at 240 μmol photon m^{-2} s^{-1} or LL at

30 μmol photon m^{-2} s^{-1} for 42 min (Fig. 2). When the plants that had been kept in the dark for more than 30 min were exposed to the constant HL, Y(I) of WT increased for about 5 min and attained a steady level, while that in LL gradually decreased. Y(II) in HL once decreased and attained a steady level, while that in LL decreased and attained the peak value at around 5 min and slightly decreased. In the *pgr5* plants, Y(I) in LL showed a transient similar kinetic to that of WT. Y(I) in HL, however, once decreased, increased to the peak at around 10 min and then decreased very slightly. Changes in Y(II) in *pgr5* were similar to those in WT. In HL, both Y(I) and Y(II) in *pgr5* were considerably lower than those in WT.

Light responses of the steady-state PSI and PSII parameters at various PPFDs

Light responses of PSI and PSII parameters obtained from chlorophyll fluorescence and P700 signals were further analyzed (Fig. 3). For energy captured by PSI pigments, the quantum yield of the PSI photochemistry, Y(I), the quantum yield of non-photochemical energy dissipation due to the donor-side limitation, Y(ND), and that of the energy dissipation due to the acceptor-side limitation, Y(NA), were measured. The fluorescence parameters measured included the effective PSII quantum yield, Y(II), the quantum yield of regulated energy dissipation, Y(NPQ), and that of non-regulated energy dissipation, Y(NO).

Y(I) and Y(II) in both WT and *pgr5* decreased with the increase in PPFD. In WT, Y(NA) was greater than Y(ND) at PPFDs less than 250 μmol photon m^{-2} s^{-1}, while, above this level, Y(NA) decreased and Y(ND) increased. In WT, with the increase in PPFD, Y(NPQ) markedly increased, while Y(NO) increased only slightly. *pgr5* showed trends very different from those of WT. Y(NA) of *pgr5* was similar to that of WT up to 100 μmol photon m^{-2} s^{-1}, but it markedly increased with further increases in PPFD, causing the drastic decrease in Y(I) in *pgr5* at PPFDs above 150 μmol photon m^{-2} s^{-1}. Y(ND) in *pgr5* was nearly zero over the entire PPFD range. These results indicate that, at high PPFDs, the electron flow through PSI in *pgr5* was limited by the acceptor-side reactions. Furthermore, the increase in Y(NPQ) was much less than that in WT, while Y(NO) markedly increased.

Effects of fluctuating light on photoinhibition of photosystems and photosynthetic electron transport

Maximum level of P700 signal (full oxidation of P700) in the dark (ΔA_{max}) and maximum quantum yield of PSII (F_v/F_m) were measured before and after the treatment with the constant HL (240 μmol photon m^{-2} s^{-1}) or the fluctuating light (alternating between HL at 240 μmol photon m^{-2} s^{-1} for 2 min and LL at 30 μmol photon m^{-2} s^{-1} for 2 min), both for 42 min (Fig. 4). After the light treatments, plants were kept in the dark for 30 min and ΔA_{max} and F_v/F_m were measured. ΔA_{max} and F_v/F_m were unchanged after the constant HL treatment

for 42 min from the levels before the treatment (Fig. 4a, c), indicating that photoinhibition of photosystems hardly occurred. Y(NA) and 1 – qL were measured in LL at 30 μmol photon m^{-2} s^{-1} for 5 min before and after the 42-min light treatment. The data obtained at the end of 5-min LL are denoted as $Y(NA)_{30}$ and $1 – qL_{30}$, respectively. After the HL treatment, both $Y(NA)_{30}$ and $1 – qL_{30}$ in *pgr5* were significantly higher than before (Fig. 4b, d), indicating some damage to the acceptor side of PSI.

WT showed small decreases both in ΔA_{max} and F_v/F_m after the treatment with the fluctuating light treatment (Fig. 4e, g). In contrast, ΔA_{max} in the *pgr5* plants after the fluctuating light treatment decreased by 38%, while F_v/F_m decreased only slightly. Although there were only small decreases in ΔA_{max} and F_v/F_m in WT, $Y(NA)_{30}$ and $1 – qL_{30}$ after the fluctuating light treatment increased by 42% and 135% (Fig. 4f, h), respectively, indicating some damage to the acceptor-side of PSI and competence of PSI in oxidizing the intersystem chain. In *pgr5*, $Y(NA)_{30}$ and $1 – qL_{30}$ increased by 94% and 332% after the fluctuating light treatment.

Do the *pgr5* plants show the CEF-PSI activity in constant light?

To investigate whether the *pgr5* plants showed CEF-PSI activity in constant light, I estimated the electron transport rate through PSI (ETR(I)) and PSII (ETR(II)) simultaneously. The photochemical quantum yield of PSI, Y(I), may be expressed as $Y(I) = Y(L_I) + Y(WWC) + Y(CEF_I)$, where $Y(L_I)$, Y(WWC), and

$Y(CEF_I)$ are the quantum yields of the LEF through PSI, the WWC, and the CEF-PSI, respectively. Similarly, the photochemical quantum yield of PSII, Y(II), may be written as $Y(II) = Y(L_{II}) + Y(WWC)$, where $Y(L_{II})$ is the quantum yield of the LEF through PSII.

To obtain directly comparable ETR(I) and ETR(II) values, I measured leaf absorptance, and estimated the share of absorbed light energy allocated to PSII (f_{PSII}). Fig. 5A shows relationships between the gross O_2 evolution in the air containing 5% CO_2 and the absorbed PPFD at low PPFDs calculated with the absorptance values, measured in four leaves each of WT and *pgr5*, respectively. When compared at the same absorbed PPFD, the O_2 evolution rates for *pgr5* leaves was always lower than that for the WT leaves, indicating the quantum yield of O_2 evolution on absorbed quantum basis was lower in the *pgr5* leaves. However, F_v/F_m values in the leaves did not differ between WT and *pgr5*. Fig. 5B shows relationships between the quantum yield of gross O_2 evolution ($Y(O_2)$) and that of PSII photochemistry (Y(II)) in the same leaves used for Fig. 5A. The slope of the line should be proportional to the share of absorbed light energy allocated to PSII (f_{PSII}). In the *pgr5*, the slope was lower by 17.3% than in the WT. The share of absorbed light energy allocated to PSII was 35 ± 3.4% in *pgr5*, while the share in WT was 47 ± 4.1%. With these values, it would be possible to calculate the absolute rates of ETR(I) and ETR(II).

In the WT plants, the ETR(I)/ETR(II) ratio was close to 1 at PPFDs below 100 μmol photon m^{-2} s^{-1}, and subsequently increased with the increase in PPFD. In contrast, the ETR(I)/ETR(II) ratio in *pgr5* was high at low PPFDs

and decreased with the increase in PPFD and eventually became close to 1 (Fig. 6). In accordance with the view accepted widely, contribution of the CEF-PSI increased with the increase in PPFD in WT, while *pgr5* showed a contrasting trend: the contribution of CEF-PSI decreased with the increase in PPFD.

The ETR(I)/ETR(II) ratio would not be correct if there were large changes in the energy share between PSII and PSI due to the state transition. To examine the contribution of the state transition on the change in the ETR(I)/ETR(II) ratio, I determined the components of non-photochemical chlorophyll fluorescence quenching, qN, from the relaxation kinetics of qN in the dark. Fig. 7 shows the extents of energy-dependent quenching (qE), state transition quenching (qT) and photoinihibitory quenching (qI) after 20 min of constant light at PPFDs of 30, 240 and 470 μmol photon m^{-2} s^{-1}. Both WT and *pgr5* leaves exhibited substantial qE, although qE in the *pgr5* leaf was much less than that in WT plants as was reported previously (Munekage et al. 2002, 2008). qT components were small compared to qE in both plants. As the measurements were carried out with red actinic light, there was no effect of chloroplast avoidance movement on apparent state transition (Cazzaniga et al. 2013). Therefore, it is unlikely that the state transition affected the ETR(I)/ETR(II) very much.

Table 1 shows the chlorophyll contents on leaf area basis in WT and *pgr5* leaves. The chlorophyll content was higher in WT than in *pgr5*. There were some differences in absorptance, reflecting the difference in the chlorophyll content. The chlorophyll *a/b* ratio in the *pgr5* leaves was greater

than that in WT leaves by 0.4.

Does the WWC in the *pgr5* plants function in constant light?

Next, I investigated whether the *pgr5* plants were able to drive the WWC. In the WWC, the electron acceptor from PSI is O_2 and the extent of the electron flow to the WWC depends on O_2 concentration (Miyake and Yokota 2000). The CEF-PSI activity has been shown to increase at low O_2 concentration, indicating suppression of the WWC by low O_2 (Arnon and Chain 1975, 1979; Scheller 1996; Makino et al. 2002). If the *pgr5* plants possessed no WWC capacity, the activity of the CEF-PSI would not be enhanced even at low O_2 concentrations. I measured the light dependence of the ETR(I)/ETR(II) ratio on the O_2 concentration in the chamber, namely at 20, 2.7 and 0% O_2 (Fig. 8). In the *pgr5* plants, the ETR(I)/ETR(II) ratios at 2.7 and 0% O_2 were higher than that at 20% O_2 when PPFD was greater than 100 μmol photon m^{-2} s^{-1}. Both WT and *pgr5* exhibited the highest ETR(I)/ETR(II) ratios at 2.7% O_2 at any PPFD below 300 μmol photon m^{-2} s^{-1}. Therefore, it is likely that the *pgr5* plants possessed the WWC capacity.

Response of the ETR(I)/ETR(II) ratio to fluctuating light

Changes in the ETR(I)/ETR(II) ratio in the fluctuating light are shown in Fig.

9. The same fluctuating light regime used for the data in Fig. 1 was used. Being consistent with the data in Fig. 6, the ETR(I)/ETR(II) ratio in the *pgr5* plants was lower in HL-period than in LL-period, whereas WT plants showed the higher ETR(I)/ETR(II) ratio in HL-period than in LL-period. In WT, ETR(I)/ETR(II) ratio was maximum at 15 s after each transfer from LL-period to HL-period, and, within 2 min, decreased slowly toward the steady-state value. The maximal value at 15 s after the transfer gradually increased with the cycle, while the steady-state value slightly decreased. In contrast, in *pgr5*, the ETR(I)/ETR(II) ratio rapidly decreased to the minimum at 15 s after the transfer from LL-period to HL-period. The minimal value gradually increased with the cycles. Also the ratio at the last data point in the HL-period increased with the cycle. The peak value in the 2 min LL-period gradually increased. In each of the LL-periods, the ratio decreased.

Effects of O_2 concentration on responses of electron transport to fluctuating light

Responses of Y(II) to the fluctuating light were measured at 2.7 and 0% O_2 (Fig. 10). In *pgr5*, Y(II) at the end of the LL-period decreased only slightly with the cycle at 2.7% O_2. The decrease in Y(II) in LL was further smaller at 0% O_2. Moreover, in contrast to the gradual decrease in Y(II) in HL-period at 20% O_2, Y(II) in HL increased with the cycle at 2.7 and 0% O_2. Similarly, in WT, Y(II) at the end of the LL-period did not decrease with the cycle at 2.7 or

0% O_2, whereas Y(II) at the end of the LL-period decreased at 20% O_2. Y(II) in the HL-period at 2.7 and 0% O_2 continued to increase with the cycle and did not reach the steady-state values within 42 min.

I assessed the degrees of photoinhibition after the treatment with the fluctuating light for 42 min at 0, 2.7 or 20% O_2 and dark treatment for 30 min (Fig. 11) using the same experimental protocol that was used for the data shown in Fig. 4. The *pgr5* plants showed little photoinhibition of PSI at 2.7 and 0% O_2 compared with that at 20% O_2 (c.f. Fig. 4e and Fig. 11a and e). The marked increases observed in $Y(NA)_{30}$ and $1 - qL_{30}$ after the light treatment at 20% O_2 were much suppressed at low O_2, although the increases were consistently observed not only in *pgr5* but also in WT.

Responses of PSII and PSI quantum yields to fluctuating light in NDH-deficient mutant

I measured the responses of an NDH-deficient mutant, *crr2-2*, to the fluctuating light under the same conditions as those use for Fig.1 (Fig. 12). The results in the *crr2-2* were almost identical to those in WT. Y(II) of *crr2-2* decreased with the cycles. The light dependence of the ETR(I)/ETR(II) ratio exhibited trends similar to those in WT (Fig. 13). Moreover, at any PPFD, the ratios at 2.7 and 0% O_2 were higher than that at 0% O_2.

2.4. Discussion

Fluctuating light as a stress factor causing photodamage

How plants use sunflecks, pulses of light at high intensity, is the topic that has attracted attention of researchers for decades (Allee 1926; Evans 1956). There have been many laboratory-based mechanistic studies as well as field-oriented studies focusing on photosynthetic responses to the fluctuating light. For instance, photosynthetic responses of the plants grown in controlled-fluctuating light and those grown in constant light were compared (Yin and Johnson 2000; Alter et al. 2012; Suorsa et al. 2012). The responses were also compared between plants grown in natural fluctuating light in a forest understory and those grown in an open field site (Knapp and Smith 1989). In most of these studies, photosynthetic responses to the single light pulse were examined. However, in natural environments, such as the forest understory, light fluctuates more frequently, as many researchers quantified (Pearcy 1983, Chazdon 1988; Vierling and Wessman 2000). Although there have been some pioneering studies (Alter et al. 2012; Suorsa et al. 2012), our knowledge of the photosynthetic responses to the fluctuating light is still poor.

It is important to choose appropriate fluctuating light regimes for studying plant responses to the fluctuating light. The light environment in the forest understory drastically changes due to sunflecs. Most sunsflecks are less than several minutes in length, and have PPFD more than several-fold that of LL-periods (Pearcy 1983; Koizumi and Oshima 1993; Vierling and

Wessman 2000). Recently, Suorsa et al. (2012) grew several mutant lines of *Arabidopsis thaliana* in the light alternately changing from low light (LL) at 50 μmol photon m^{-2} s^{-1} for 5 min to high light (HL) at 500 μmol photon m^{-2} s^{-1} for 1 min and successfully elucidated a role of the PGR5 protein in acclimation to the fluctuating light. In the present study, I used a fluctuating light regime with the same durations of HL and LL. The duration I adopted was 2 min because the photosynthetic parameters most drastically change upon the change from the LL- to HL-period in the first 2 min in *A. thaliana* plants. Thus, the light fluctuation in the 2 min intervals would subject the plants to the most stressful situation. Next, I chose intensity of the HL. The HL at 240 μmol photon m^{-2} s^{-1} was strong enough, but induced no photoinhibition when given continuously. The present results indicate that the fluctuating light I adopted was suitable for analysis of the effects of fluctuating light on photoinhibition. The results clearly showed that light fluctuation itself is a very effective stress factor causing photodamage. I propose the term 'fluctuating light photoinhibition' and the target is mainly PSI as has been already indicated by the pioneering studies (Munekage et al. 2002, 2008; Suorsa et al. 2012).

Effects of short-term fluctuating light on photosynthetic electron transport system

The decreases in the photochemical quantum yield of PSII, Y(II), of the WT

and *pgr5* plants occurred soon after the start of the fluctuating light treatment. Y(II) of *pgr5* decreased more drastically (Fig. 1). Moreover, these plants showed photoinhibition of PSI by the fluctuating light treatment. In particular, the extent of PSI photoinhibition in *pgr5* was marked. It should be noted that this was not the result of the long-term effect of the fluctuating 'growth' light (Suorsa et al. 2012) but that of the treatment for a short period. It has been reported that PSI of *pgr5* was sensitive to light (Munekage et al. 2002, 2008). In the present study, I found that PSI activity in *pgr5* was limited by the acceptor-side reactions: Y(NA) was higher than Y(ND) over the entire PPFD range examined (Fig. 3).

What component/event in the PSI acceptor-side did limit photochemical reaction and thereby cause PSI photoinhibition? The crucial difference between the fluctuating light and constant light was that fluctuating light included LL-period, during which photosynthetic activities were lower than those at HL-period. When leaves were in the LL, various reactions that had occurred in response to HL, including the de-epoxidation of violaxanthin and protonation of the PsbS protein, would be relaxed to some extents. In HL-period, the thylakoid lumen acidification would not be enough for down-regulation of LEF via the photosynthetic control of plastoquinol re-oxidation at Cyt b_6f complex (Rott et al. 2011; Suorsa et al. 2012), particularly in *pgr5*. Therefore, especially in *pgr5*, when the every HL-period started, the thylakoids in the more or less relaxed state would cause a gush of electron flow to PSI leading to prompt reduction of electron acceptors, O_2 photoreduction, and formation of ROS. From the preceding studies (Sonoike

1996; Sonoike et al. 1997; Choi et al. 2002), it is clear that the photoinhibition of PSI involves ROS and thereby require O_2.

It is noteworthy that the PSI photoihibition occurred even in the WT plants by the fluctuating light; the photodamage by the fluctuating light is not a phenomenon specific only to *pgr5*.

The effective responses avoiding photoinhibition to the fluctuating light

The large Y(NA) means the high level of PSI acceptor-side limitation. This is not necessarily directly associated with photoinhibition of PSI. As clearly shown in Fig. 4, *pgr5* treated in continuous HL showed little photoinhibition of PSI, although $Y(NA)_{30}$ and $1 - qL_{30}$, measured at the end of the illumination of 30 μmol photon m^{-2} s^{-1} for 2 min, just after the constant HL for 42 min, were very high. On the other hand, when fluctuating light was applied for 42 min, PSI photoinhibition occurred in both WT and *pgr5*, in addition to the increases in $Y(NA)_{30}$ and $1 - qL_{30}$. The extent of the damage was markedly greater in *pgr5*. The large difference in the PSI photoinhibition between the WT and *pgr5* plants indicates that WT had mechanisms to cope with rapid light fluctuations. I hypothesized that this would be related to photosynthetic alternative electron flows interacting with PSI because PSI was firstly photoinhibited in the fluctuating light. Previous works reported that, in *pgr5*, the CEF-PSI via the putative ferredoxin-dependent quinone reductase (FQR)

was impaired (Munekage et al. 2002, 2004; DalCorso et al. 2008). However, some other studies reported that *pgr5* possessed CEF-PSI capacity (Nandha et al. 2007; Joliot and Johnson 2011). I tried to assess the activities of the alternative electron flows in *pgr5*.

Several methods have been used to quantify the rate of the CEF-PSI (for a review, see Kramer et al. 2004b). In LEF, the rates of electron transfer through PSII should equal that through PSI (Klughammer and Schreiber 1994) or the Cyt b_6f complex (Klughammer and Schreiber 1994; Sacksteder and Kramer 2000). Thus, the relationship between some factors associated with the electron flow and the LEF should be changed when the activity of the CEF-PSI becomes substantial. In turn, from the increased ratios of these factors to LEF, the rate of the CEF-PSI would be assessed. The possible factors would include the proton to electron stoichiometry (Sacksteder et al. 2000), electrochromic shift of carotenoid pigments due to the electric field formation across the thylakoid membrane (Joliot and Joliot 2002, 2005; Joliot et al. 2004; Sacksteder and Kramer 2000), and the proportion of overall photosynthetic energy storage assessed by the photoacoustic method (Herbert et al. 1990; Joet et al. 2002). More directly, measurements of post-illumination re-reduction kinetics of $P700^+$ after red + far-red actinic light (Fan et al. 2007), or after a far-red illumination (Maxwell and Biggins 1976; Joet et al. 2002; Chow and Hope 2004), have been conducted. The transient rise in the fluorescence level after turning off the actinic light has been also measured as a parameter reflecting the activity of CEF-PSI (Asada et al. 1993; Burrows et al. 1998). However, it is not feasible

to measure the absolute rate of the CEF-PSI in situ with these techniques. Instead, I used the ETR(I)/ETR(II) ratio as an indicator of the CEF-PSI activity (Fig. 6). Because the linear electron transport rate through PSII can be quantified (Genty et al. 1989), if the ratio is properly obtained, the rate through PSI may be quantified. For this purpose, I measured leaf absorptance and the share of absorbed light energy allocated to PSII (f_{PSII}) (Table 1). Very recently, Kou et al. (2013) estimated the activity of PSI-CEF at saturating CO_2 based on measurements of the O_2 evolution rate and PSI quantum yields. However, they did not measure f_{PSII}.

Solving the equation, $Y(O_2) = I_A \times f_{PSII} \times Y(II)/4$, where I_A is the absorbed PPFD (Genty et al. 1989), I obtained the share of absorbed light energy allocated to PSII. In spite of the fact that the plants were grown in constant illumination, the share in *pgr5* was 35%, while WT showed almost equal sharing of light energy between PSI and PSII (Fig. 5B). Furthermore, the contribution of the state transition to the energy share was small over the range from low- to high-PPFD in both plants (Fig. 7) in agreement with the studies reporting that the state transitions in higher plants were not marked (Pesaresi et al. 2011). These results indicate that the share of light energy allocation to PSI was much greater than that to PSII in *pgr5*. In the light response curve shown in Fig.3, Y(I) in *pgr5* started to decrease from very low PPFDs, whereas that in WT was relatively high up to PPFD of ca. 250 μmol photon m^{-2} s^{-1} and started to decrease with further increase in PPFD. In *pgr5*, a limitation of electron flow through PSI due to this decrease in Y(I) at low PPFD would be compensated by the increase in the share of absorbed light

energy allocated to PSI. This was also supported by the fact that the chlorophyll *a*/*b* ratio was greater in the *pgr5* leaves than that in WT by 0.4. The previous studies reported that growth of *pgr5* was similar to that of WT in both low light (Munekage et al. 2008) and moderate light (Suorsa et al. 2012). However, in fact, energy sharing between two photosystems and the composition of chlorophyll proteins would be markedly changed in *pgr5*. The calculation of ETR using properly measured f_{PSII} may be a useful method to estimate CEF-PSI.

When white light was used as the actinic light, maximum $Y(O_2)$ values for non-stressed leaves of C_3 plants approached 0.105 (Björkman and Demmig 1987). In this study, $Y(O_2)$ decreased with the absorbed PPFD at low PPFDs because red light was used for the actinic light. However, when the maximum $Y(O_2)$ was obtained by extrapolating the line in Fig. 5B to Y(II) of 0.81, the value for WT was 0.09, a value within the range of the data for C_3 species (Björkman and Demmig 1987).

From the light energy allocation to PSI and the changes in the ratio of ETR(I)/ETR(II) measured in the constant- and fluctuating-light, I suggest that *pgr5* plants possessed the CEF-PSI activities because the ratios at low PPFDs and LL in the fluctuating light were far above 1 (Figs. 6 and 9). These results also indicate that, under the growth light conditions at a PPFD of 100 μmol photon m^{-2} s^{-1}, *pgr5* drove CEF-PSI continuously (Nandha et al. 2007). On the other hand, the ETR(I)/ETR(II) ratio in WT increased with the increase in PPFD. This suggests that the CEF-PSI not only function during photosynthetic induction (Makino et al. 2002; Joliot and Joliot 2002, 2005

2006; Fan et al. 2007) but also at the steady-state conditions in high light.

There are some O_2-dependent pathways besides the WWC, and they may contribute to the photodamage by the fluctuating light. Photorespiration is one of the O_2-dependent pathways. When CO_2 and O_2 concentrations were 800 ppm and 20% in the leaf chamber, respectively, where the effect of photorespiration was suppressed to a considerable extent, Y(II) of WT and *pgr5* showed responses similar to those at 390 ppm and 20%. Plastid terminal oxidase (PTOX) is also proposed to be associated with O_2-consumption, in the reaction called chlororespiration. The PTOX is a plastoquinol oxidase, and is able to transfer electrons from PQ to O_2. Thus, chlororespiration can be a source of ROS generation. However, PTOX is suggested to play an important role in chloroplast biogenesis rather than in stress responses (Rosso et al. 2006). Furthermore, in plants grown under normal conditions, PTOX is present at about only 1% of the level of the D1 protein that houses the PSII reaction center (Lennon et al. 2003). Therefore, the contributions of photorespiration and chlororespiration to the photodamage caused by the fluctuating light would be small, if any.

In both the constant- and fluctuating-light, *pgr5* appeared to show WWC activities (Fig. 8). When light dependence of the ETR(I)/ETR(II) ratio was measured at 2.7 or 0% O_2, the ratios in *pgr5* at PPFD above 100 μmol photon m^{-2} s^{-1} were greater than those measured at 20% O_2. This suggests that, at least some fraction of electrons that flowed through the WWC at 20% O_2, would flow through the CEF-PSI at low O_2 concentrations, resulting in the increases in the ETR(I)/ETR(II) ratio. The ETR(I)/ETR(II) ratios in WT

and *pgr5* were highest at 2.7% O_2 rather than at 0% O_2 for all the PPFD levels examined (Fig. 8). Reasons for this are unknown.

For WT plants in the fluctuating light, the ETR(I)/ETR(II) ratio in the HL-periods rapidly increased immediately after each transition from LL- to HL-period, attained the maximal levels, then decreased and attained the steady-state values within HL-periods. Although the steady-state values decreased in a stepwise fashion with the cycle, the maximal levels were almost constant. In contrast, in *pgr5*, the ratio rapidly decreased immediately after each transition from LL- to HL-period and then attained the minimum levels within the HL-periods. Under these conditions, PSI of *pgr5* would be more sensitive to the damage due to the fact that PSI capacity was not able to manage the gush of the electron flow caused by the rapid increase in PPFD. However, at low O_2 concentrations, Y(II) in LL-period in fluctuating light did not decrease with the cycles (Fig. 10), and no photoinhibition of PSI occurred after the light treatment (Fig. 11). These results indicate that an increase in the activity of the CEF-PSI at low O_2 concentrations lead to relaxation of the acceptor-side limitation of PSI, resulting in acceleration of the linear and/or the other electron flows. Therefore, I conclude that the CEF-PSI is essential to efficiently cope with the rapid increase in PPFD and preventing photoinhibition of PSI caused by the fluctuating light. Furthermore, my data indicate that the CEF-PSI could be regulated by O_2. The enhancement of the CEF-PSI by low O_2 is probably attributable to suppression of the electron flow to O_2 at low O_2. As the activity of the CEF-PSI cannot be properly regulated in *pgr5*, considerable electrons inevitably flow to O_2, leading to ROS formation

and thereby PSI photoinhibition. From these, it is suggested that, in WT, electron flow to O_2 can be controlled by regulating engagement of alternative electron flows including CEF-PSI in a way that the photooxidative damage is minimized even at 20% O_2.

In *A. thaliana*, NDH-CEF has been suggested to play a complementary role, since the NDH-CEF is not essential for photosynthesis at least under ordinary laboratory conditions, and NDH-deficient mutants of *A. thaliana* grow similarly to WT (Munekage et al. 2002, 2004; Okegawa et al. 2008). I measured the responses of an NDH-deficient mutant, *crr2-2*, to the fluctuating light under the same conditions as those use for Fig.1 (Supplementary Fig. S3). The results in the *crr2-2* were almost identical to those in WT; Y(II) decreased with the cycles showing similar changes in the ETR(I)/ETR(II) ratio, and PSI was slightly photoinhibited by the fluctuating light treatment. The light dependence of the ETR(I)/ETR(II) ratio exhibited trends similar to those in WT. Moreover, at any PPFD, the ratios at 2.7 and 0% O_2 were higher than that at 0% O_2 (Supplementary Fig. S4). Thus, I conclude that the NDH-CEF would not contribute to response to the fluctuating light. It is noteworthy, however, that ETR(I)/ETR(II) ratios in WT and *pgr5* at lowest two PPFD levels were somewhat greater than those at 35 μmol m^{-2} s^{-1} (Fig. 8). This was not the case in *crr2-2*, although I did not measure the ratios at very low PPFDs for *crr2-2*. These differences may indicate that NDH-CEF in WT and *pgr5* operated at very low PPFDs as suggested for *Oryza sativa* (Yamori et al. 2011) and *Marchantia polymorpa* (Ueda et al. 2012). It is necessary to conduct detailed measurements including

f_{PSII} with *crr2-2*.

Concluding remarks and future scopes

PSI of the *pgr5* plant was sensitive as previously reported (Munekage et al. 2008) due to the large acceptor-side limitation of PSI. *pgr5* was particularly sensitive to the fluctuating light, and showed marked photoinhibition of PSI. In this study, I clearly elucidated that *pgr5* can drive CEF-PSI in low light. Namely, *pgr5* not only possesses the CEF-PSI capacity (Nandha et al. 2007) but actually drives the CEF-PSI at low PPFDs. However, its capacity dramatically decreases with the increase in the PPFD, supporting the view that the PGR5 protein is involved in the redox control of PSI (Nandha et al. 2007).

The general message of this study is that the CEF-PSI is essential for effective responses to the drastic light fluctuation. When plants are exposed to drastic fluctuation in PPFD in the field, the plant would activate the CEF-PSI more than the WWC to accommodate the electron flows and thereby avoid the risk of photo-oxidative damage. I also found that the fluctuation in PPFD is a potent stress factor, even when the PPFD level in the HL-periods is moderate.

Plants grown in the forest understory are exposed to drastic fluctuation in PPFD. If they are able to acclimate to such the fluctuating light conditions, one of the mechanisms would be an enhancement of the ability of

appropriate regulation of the activity of the CEF-PSI in response to light fluctuation. This would be achieved by the increase in the proportion of the PSI complex with the PGR5 protein. Plants may be able to avoid photodamage to PSI by altering the ratio of two photosystems in the thylakoid membranes (Suorsa et al. 2012, Yin and Johnson 2000, Jahns and Junge 1992) as observed in *pgr5* grown in the constant light in this study. I am currently examining whether the photosynthetic apparatus in WT acclimates to the drastically fluctuating growth light to actually become resistant to the fluctuating light.

2.5. Table

Table 1. Chlorophyll *a* + *b*, chlorophyll *a/b* in thylakoids and leaf absorptance in the WT and *pgr5* leaves.

Genotype	Chl *a* + *b* (mg m^{-2})	Chl *a*/*b*	Leaf absorption
WT	228 ± 23	3.38 ± 0.007	0.837 ± 0.0521
pgr5	190 ± 10 *	3.73 ± 0.009 *	0.823 ± 0.0742 *

Plants were grown at 90-100 μmol m^{-2} s^{-1} in a short-day photoperiod (8 h of light, 16 h of dark) for 55 d. Means ± SD (n = 3 to 5) are shown. *P < 0.005 (t-test, WT vs. *pgr5*). Light absorptance was measured with an integrating sphere. The light from the Björkman-type lamp passing through a 635 nm red filter was used.

2.6. Figures

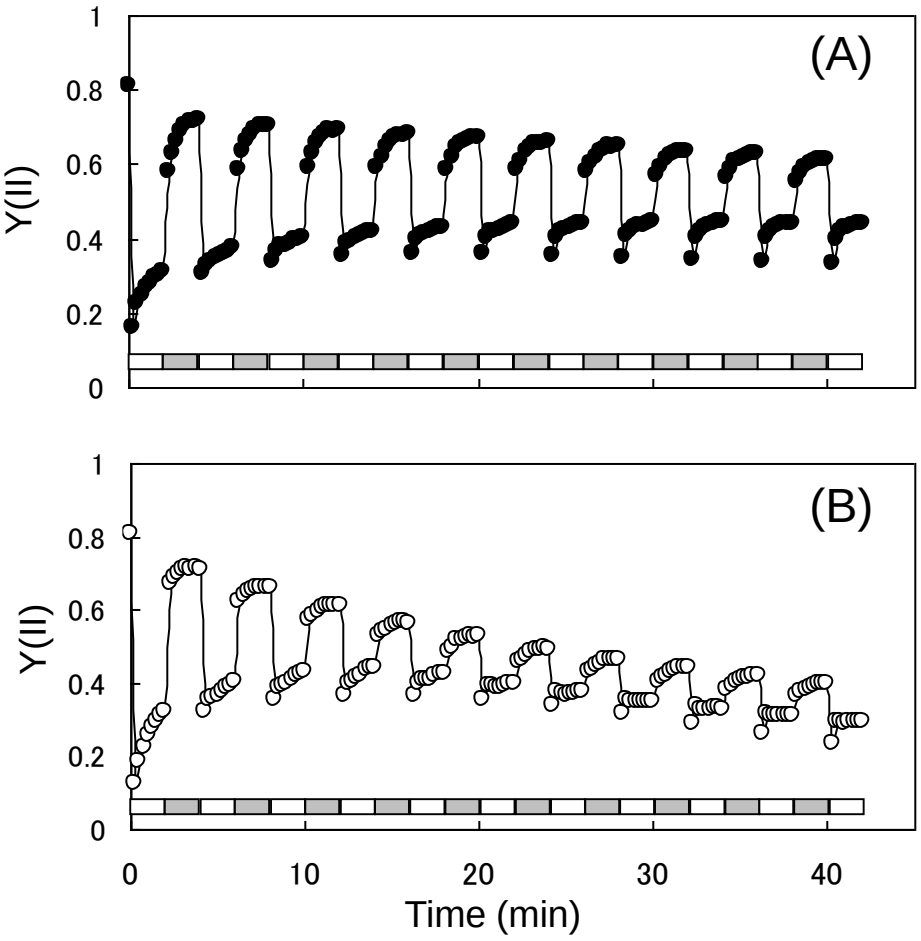


Figure 1. Response of photochemical quantum yield of PSII (Y(II)) of the WT (A) and *pgr5* (B) plants to the fluctuating light. The plants were grown in a constant moderate light (100 μmol photons m^{-2} s^{-1}) for 8 h per day. The light alternating between HL at 240 μmol photons m^{-2} s^{-1} for 2 min (open bars) and LL at 30 μmol photons m^{-2} s^{-1} for 2 min (grey bars) was applied to the leaf after the dark treatment for 30 min. The leaf lamina was sandwiched in a chamber. The air in the chamber contained 20% O_2 and 390 ppm CO_2. Each data point represents the mean (n = 5 to 6).

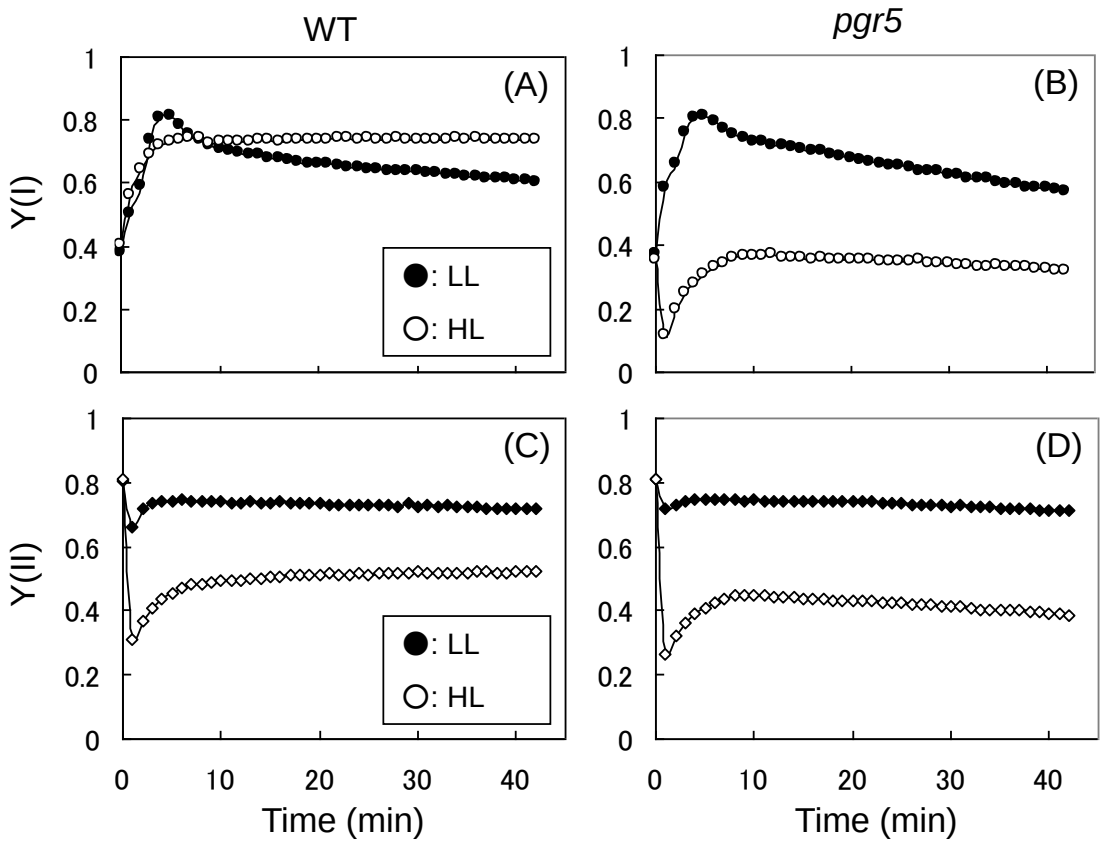


Figure 2. Changes in the quantum yield of PSI (Y(I)) and PSII (Y(II)). (A) and (B), Y(I); (C) and (D), Y(II). Continuous light at PPFD of 240 μmol photons m^{-2} s^{-1} (open symbols, HL), or at PPFD of 30 μmol photons m^{-2} s^{-1} (closed symbols, LL) was applied for 42 min after the 30 min dark treatment. Measurements were made at 20% O_2 and 390 ppm CO_2. Each data point represents the mean (n = 4).

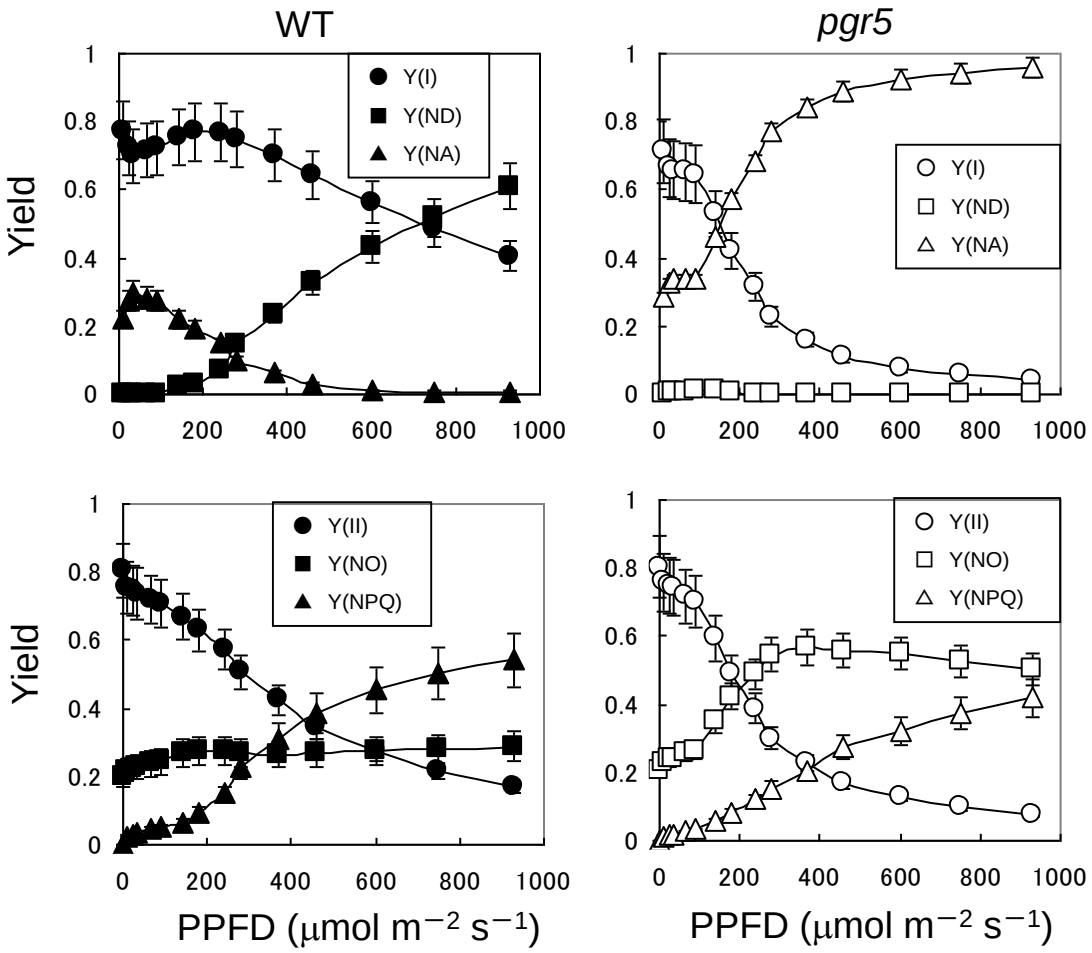


Figure 3. Changes in the photosynthetic quantum yields of PSI and PSII with PPFD of the constant light in WT (closed symbols) and *pgr5* (open symbols). For energy captured by PSI pigments, the quantum yield of the PSI photochemistry, Y(I) (circle), the quantum yield of non-photochemical energy dissipation due to the donor-side limitation, Y(ND) (square), and that of the energy dissipation due to the acceptor-side limitation, Y(NA) (triangle), are indicated. The fluorescence parameters, the effective PSII quantum yield, Y(II) (circle), the quantum yield of regulated energy dissipation, Y(NPQ) (triangle), and that of non- regulated energy dissipation, Y(NO) (square) are shown. Measurements were made at 20% O_2 and 390 ppm CO_2. The values represent the mean ± SD (n = 4 to 6).

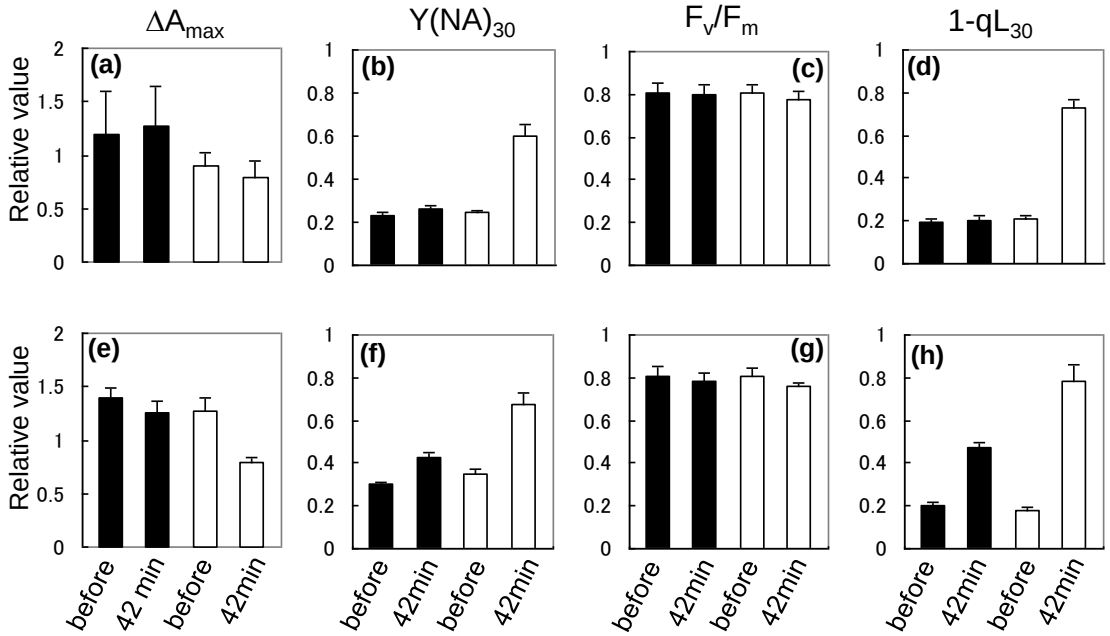


Figure 4. Effects of constant high light (a, b, c and d) and fluctuating light (e, f, g and h) on changes in photosynthetic parameters in leaves of WT (solid bars) and *pgr5* (open bars). Following the light treatments for 42 min and dark treatment for 30 min, functions of the PSI and PSII reaction centers were determined as ΔA_{max} and F_v/F_m. $Y(NA)_{30}$ and $1 - qL_{30}$ were measured at the end of the low light treatment at PPFD of 30 μmol photons m^{-2} s^{-1} for 2 min just after the light treatments for 42 min. Measurements were made at 20% O_2 and 390 ppm CO_2. Error bars represent the SD (n = 6 to 8).

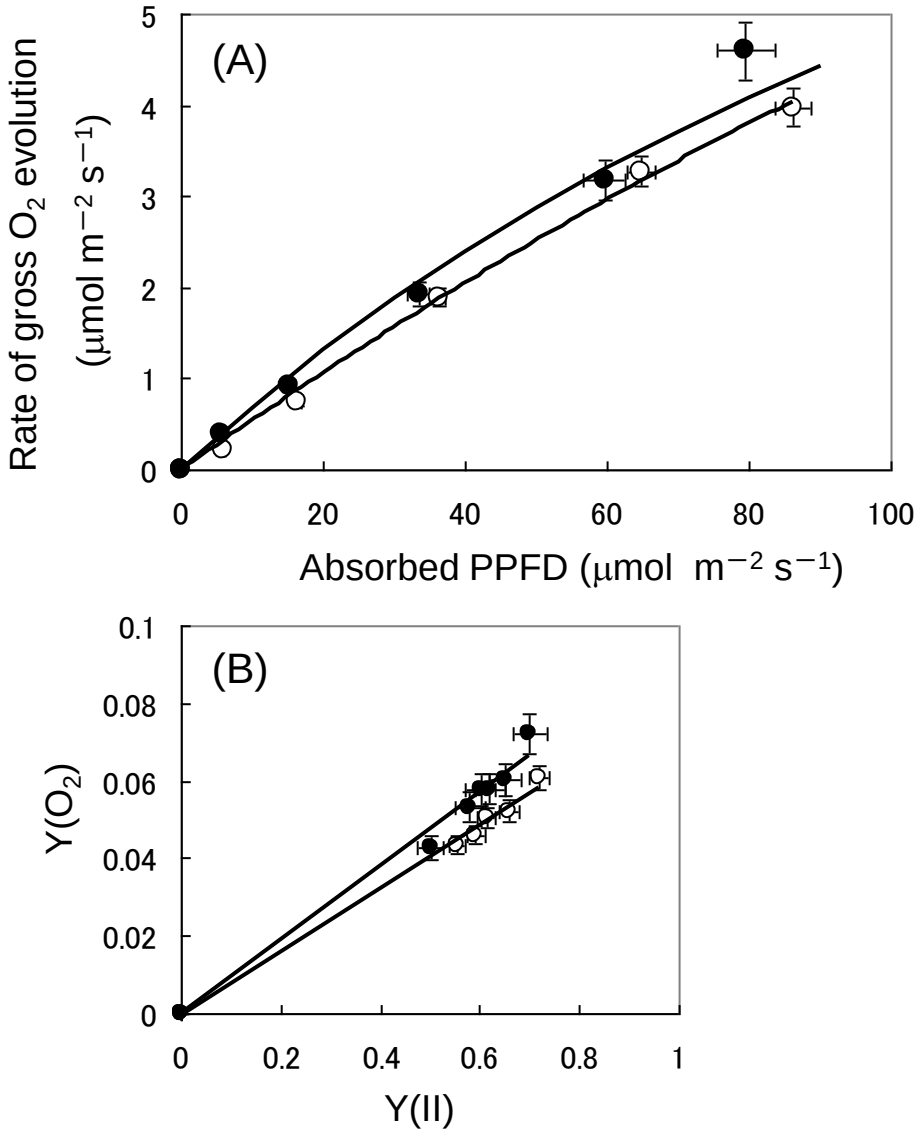


Figure 5. Estimation of the share of absorbed light energy to PSII. (A) Light-response curve of the photosynthetic O_2 evolution in the leaf discs at low PPFDs. The rate of gross O_2 evolution was plotted against absorbed PPFD Fitted hyperbolic functions through the origin are shown. (B) The relationship between the quantum yield of O_2 evolution, $Y(O_2)$, and the photochemical yield of PSII,Y(II). Closed circle; WT, open circle; *pgr5*. Error bars represent the SD (n = 4 to 6). Regression lines through the origin are shown.

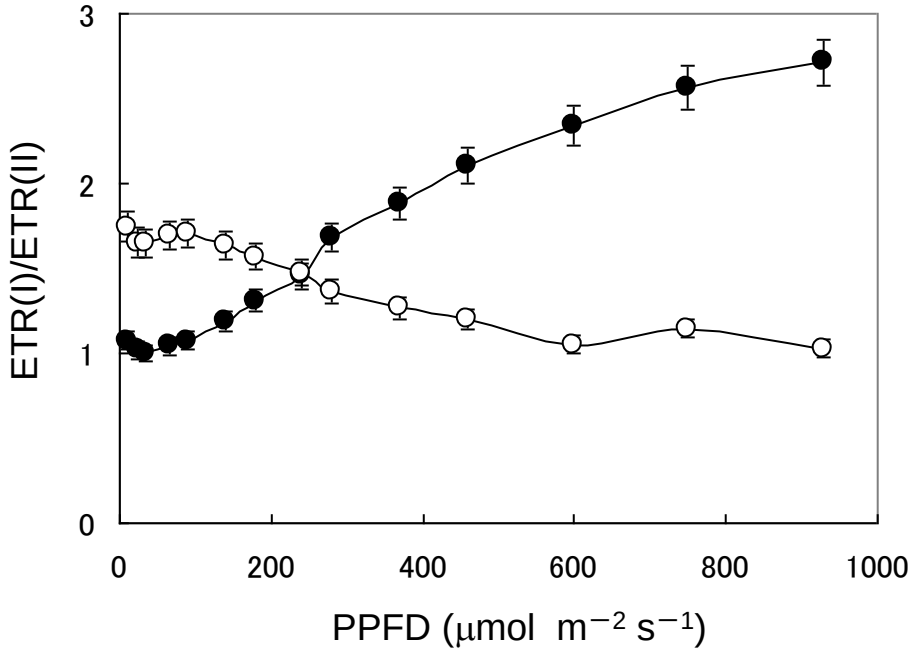


Figure 6. Changes in the ratio of ETR(I)/ETR(II) in WT (closed circle) and *pgr5* (open circle) leaves as a function of PPFD of the constant light. Y(I) and Y(II) were measured as in Figure 3. Measurements were made at 20% O_2 and 390 ppm CO_2. The values represent the mean ± SD (n = 4 to 6).

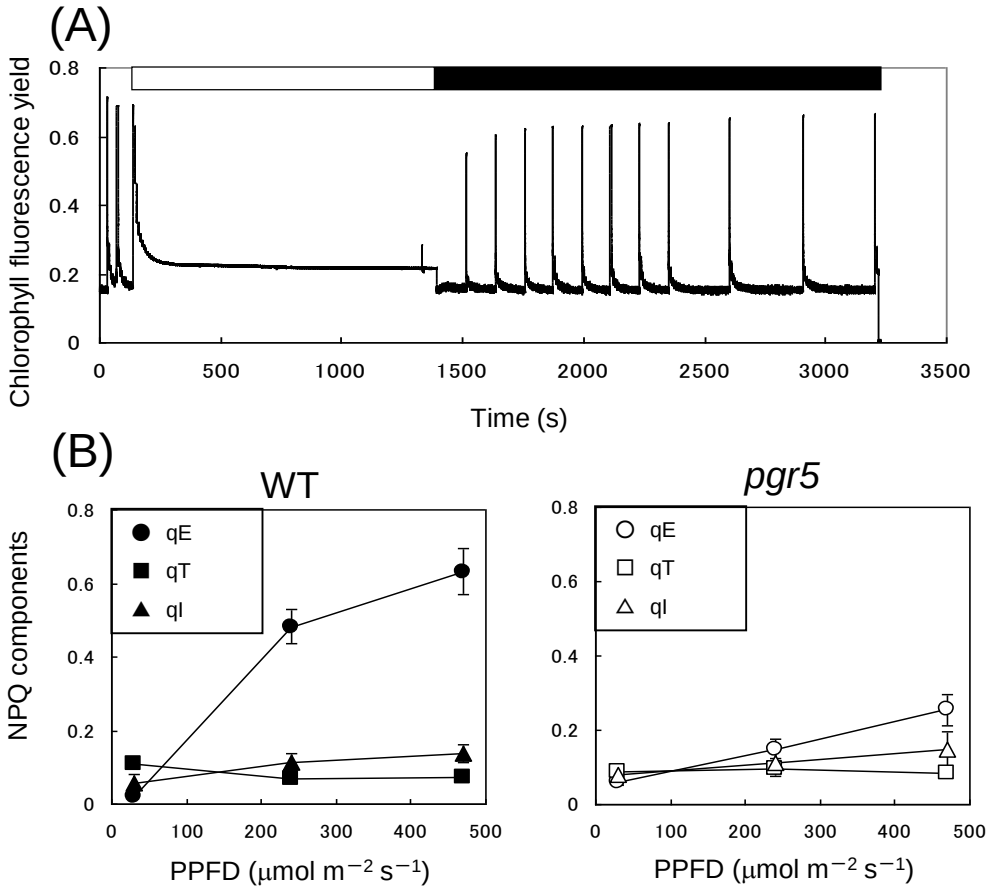


Figure 7. Dissection of NPQ into the energy-dependent quenching (qE), the state transition (qT) and the photoinhibition (qI). (A) A typical Chl fluorescence trace obtained with a WT leaf after 30 min dark adaptation. The trace shows the changes in fluorescence yield during (white bar), and after turning off the actinic light (black bar). (B) Components of non-photochemical chlorophyll fluorescence quenching in WT and *pgr5*. The different components of NPQ were derived from semi-logarithmic plots of the dark relaxation of F_v after the light treatment at three PPFDs of 30, 240 and 470 μmol photons m^{-2} s^{-1}. Energy-dependent quenching (qE; circle) was attributed to the fast phase, quenching by state transition (qT; square) to the medium phase and photoinhibitory quenching (qI; triangle) to the slow phase of relaxation. The quenching components were calculated from the amplitude of the respective phases considering the relationship $(1 - qN) = (1 - qE) \times (1 - qT) \times (1 - qI)$. Measurements were made at 20% O_2 and at 390 ppm CO_2. The values represent the mean ± SD ($n = 3$).

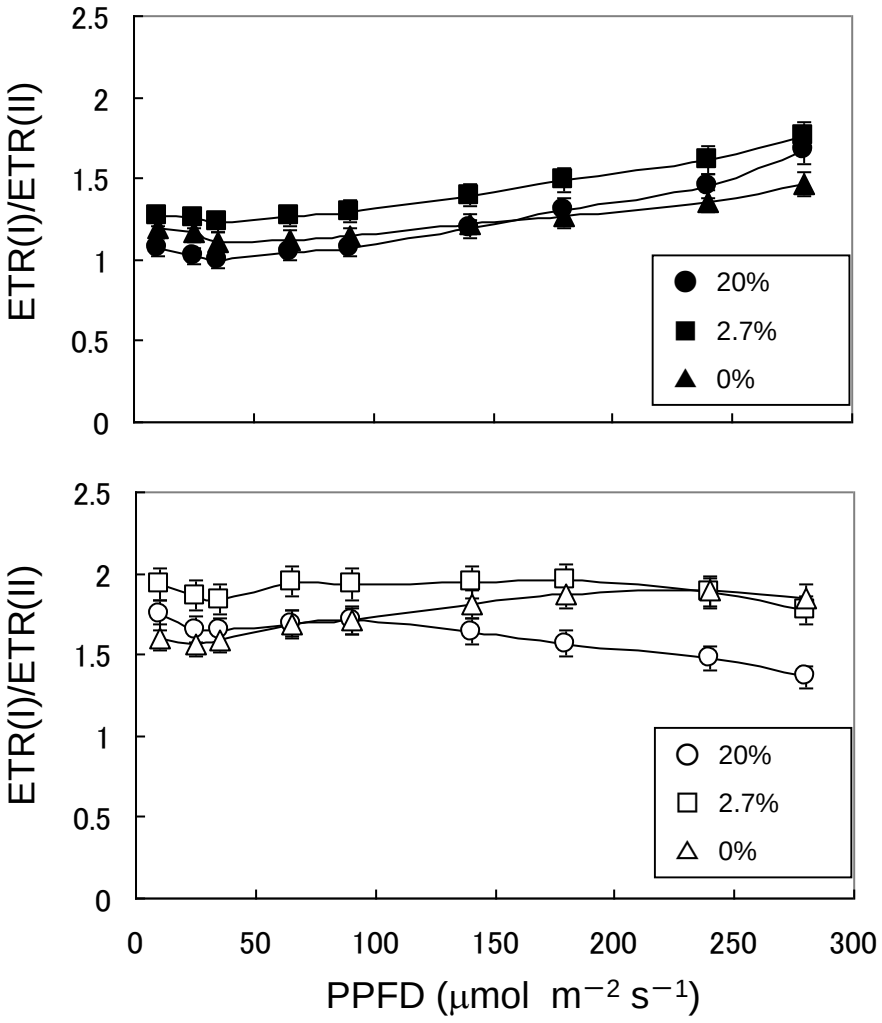


Figure 8. Light intensity dependence of the ETR(I)/ETR(II) ratio in 20, 2.7 and 0 % O_2 in the WT (closed symbols) and *pgr5* (open symbols) leaves. Measurements were made for the PPFDs ranging from 0 to 280 μmol photons m^{-2} s^{-1} and at CO_2 concentration of 390 ppm. The values represent the mean ± SD (n = 3).

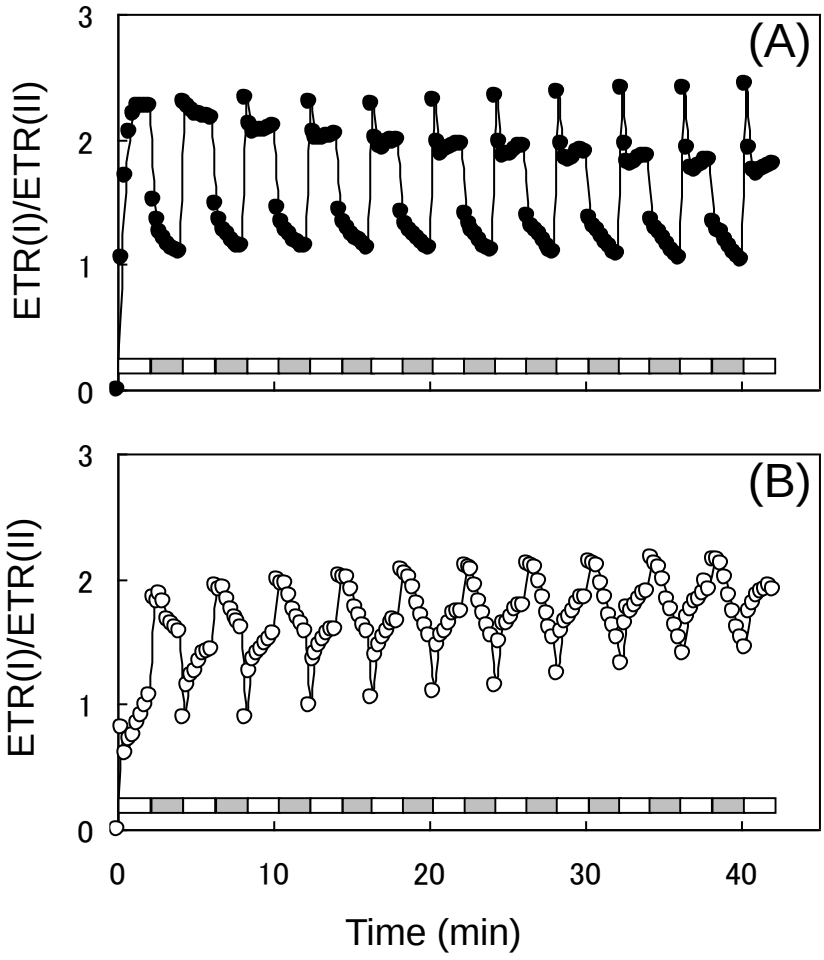


Figure 9. Changes in the ratio of ETR(I)/ETR(II) in the WT (A) and *pgr5* (B) leaves in the fluctuating light. The same light treatment protocol for Figure 1 was used. Measurements were made at 20% O_2 and 390 ppm CO_2. The values represent the mean ± SD (n = 5 to 6).

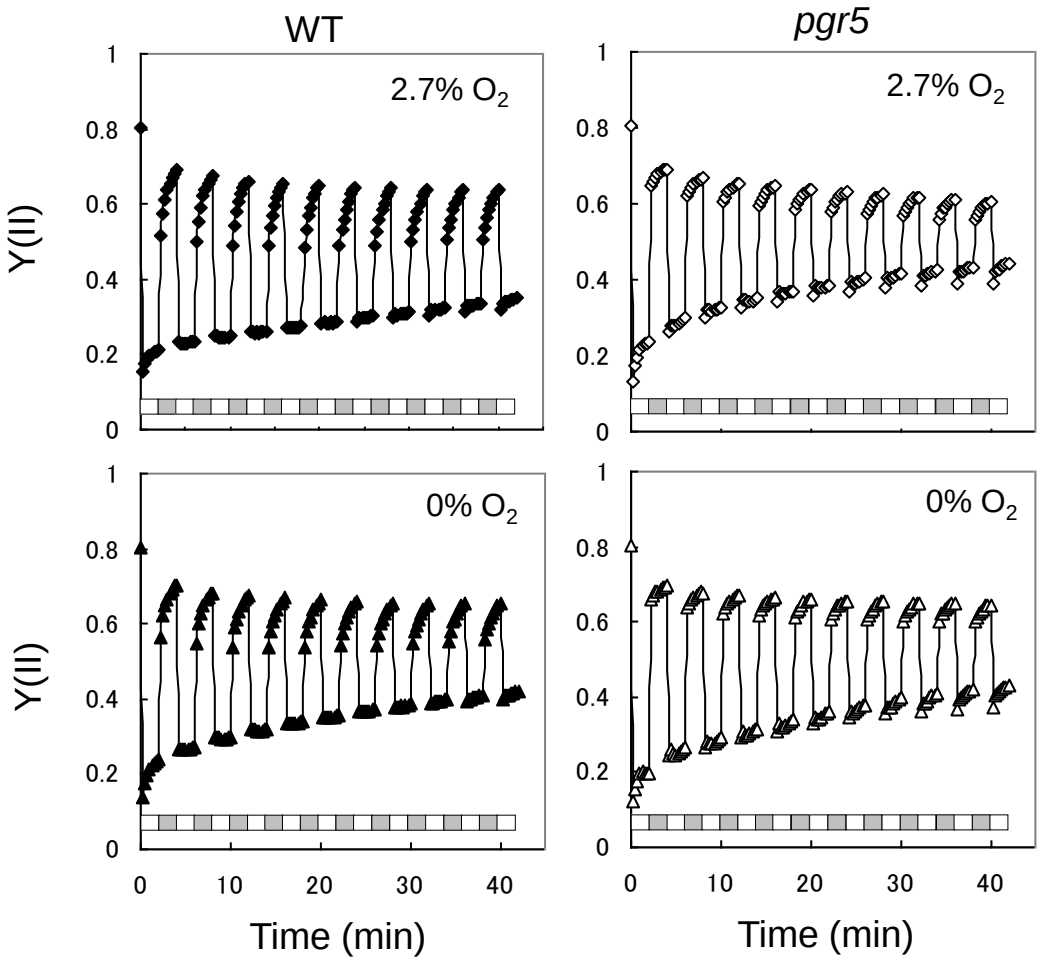


Figure 10. Effects of low O_2 concentrations (2.7%; diamond, 0% triangle) on responses of Y(II) to the fluctuating light in WT (closed symbol) and *pgr5* (open symbol). The fluctuating light treatment was the same that used for Figure 1. Measurements were made at 390 ppm CO_2. The values represent the mean, $n = 4$ to 6.

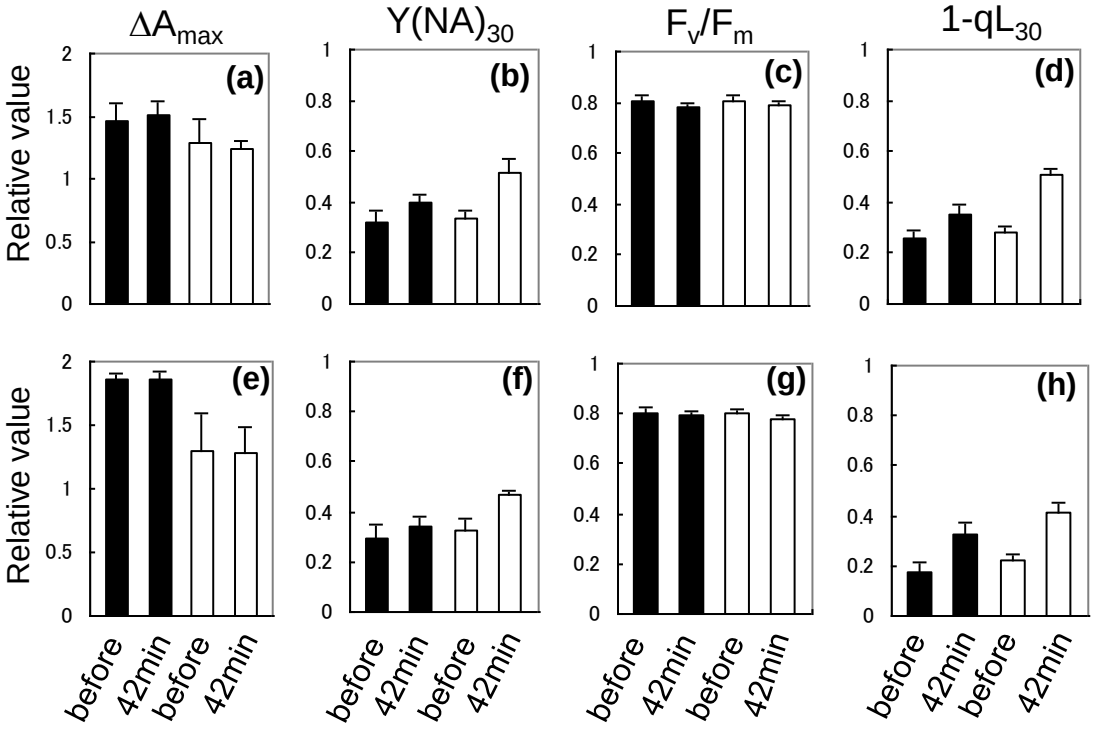


Figure 11. Effects of low O_2 concentrations (2.7%; a, b, c and d, 0%; e, f, g and h) on changes in the photosynthetic parameters after the fluctuating light treatment in WT (black bar) and *pgr5* (white bar). Measurements were made in the same manner that used for Figure 4 and at 390 ppm CO_2. Error bars represent the SD (n = 4 to 8).

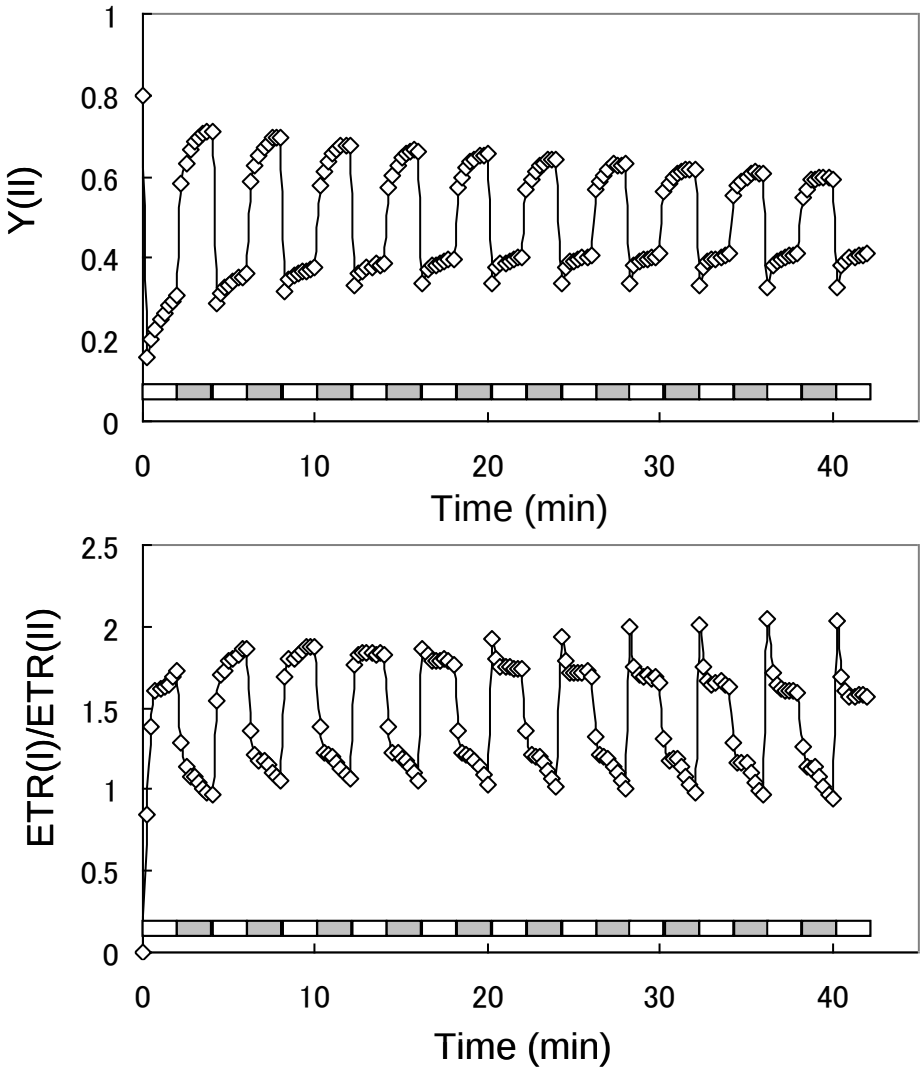


Figure 12. Response of Y(II) (A) and ETR(I)/ETR(II) ratio (B) in *crr2-2* leaves to the fluctuating light. The plants were grown in a constant moderate light (100 μmol photons m^{-2} s^{-1}) for 8 h per day. The same light treatment protocol for the data in Figure 1 was used. The air in the chamber contained 20% O_2 and 390 ppm CO_2. Value of 0.5 was used as the share of absorbed light energy allocated to PSII. The values represent the mean ± SD (n = 3).

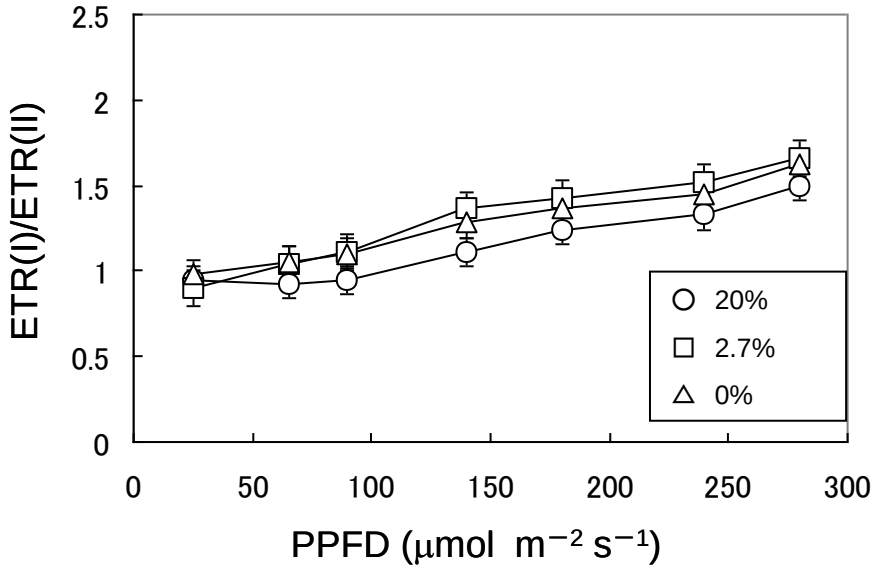


Figure 13. Light intensity dependence of the ETR(I)/ETR(II) ratio in 20, 2.7 and 0 % O_2 in the *crr2-2* leaves. Measurements were made for the PPFDs ranging from 0 to 280 μmol photons m^{-2} s^{-1} and at CO_2 concentration of 390 ppm. The values represent means ± SD (n = 3).

CHAPTER 3

Effects of fluctuating light on photoinhibition of photosystems I and II, and regulation of the photosynthetic electron transport system in *Arabidopsis thaliana*

3.1. Introduction

Photosynthetic electron transport primarily occurs via a linear pathway, in which electrons flow from water via PSII and the cytochrome *b*$_6$*f* complex (the Cyt *b*$_6$*f* complex) to PSI and reduce $NADP^+$ to NADPH. The linear electron flow (LEF) generates the transmembrane electrochemical potential difference of H^+ (ΔpH), through water splitting by PSII in the thylakoid lumen and translocation of H^+ across the thylakoid membrane by the Q cycle. The electrochemical potential difference thus produced drives the H^+-ATPase to produce ATP. ΔpH also drives ΔpH-dependent heat dissipation system, which can be measured fluorometrically as the non-photochemical quenching (NPQ).

When plant leaves receive excess light energy over the capacity for photosynthesis, the photosynthetic rate of the leaves is decreased. This phenomenon is called photoinhibition (Kok 1956; Jones and Kok 1966). Photoinhibition is due mainly to inactivation of PSII. Absorbed light energy that is neither utilized by photosynthesis nor dissipated by photoprotective mechanisms accelerates photodamage to PSII (Demmig-Adams et al. 1996). In healthy leaves under moderate conditions, the damaged PSII is rapidly repaired via the D1 protein turnover cycle, and thereby the rate of photosynthesis hardly decreases. In contrast, under stressful conditions, when excess light energy increases, the damage rate of PSII exceeds the repair rate of PSII, leading to photoinhibition, which can be measured fluorometrically as the reduction of the maximum level of functional PSII (F_v/F_m).

Plants have various photoprotective mechanisms to protect PSII from photoinhibition. These mechanisms involve changes in the light harvesting capacity, share of excitation transfer to PS II relative to PSI, and the enzymatic activities associated with the PSII turnover cycle. NPQ is a main mechanism of the photoprotection, which contributes to down-regulation of the photosynthetic electron transport in chloroplast by regulating light-harvesting capacity. Heat dissipation of excess excitation energy by NPQ prevents over-acidification of the thylakoid lumen and decreases the damage to PSII (Niyogi 2000; Vass 2011).

In contrast to PSII that is highly susceptible to photodamage, it had been widely believed that PSI would be efficiently protected against photodamage. While PSI has a tolerance for typical high light stress, it is sensitive to photodamage under certain conditions such as chilling temperatures. In *Cucumis sativus*, a chilling sensitive plant, chilling of leaves in moderate light gives damage to PSI with little damage to PSII (Terashima et al. 1994; Sonoike et al. 1995; Sonoike et al. 1997; Sonoike 2011). Photoinhibition of PSI to the extent similar to that of PSII has been reported in chilling-tolerant plants such as *Arabidopsis thaliana* (Zhang and Scheller 2004). PSI photoinhibition has been also shown in other plants, such as potato (Havaux and Davaud 1994), winter rye (Ivanov et al. 1998) and barley (Tjus et al. 1999). Recently, it was reported that PSI was photoinhibited by growing the plants in fluctuating light (Suorsa et al. 2012). This PSI photoinhiition occurs even by a short exposure to moderate fluctuating light for a short term of less than 1 h as has been shown in Chapter 2. These observations suggest

that light fluctuation is a potent stress factor especially for PSI, and that plants require mechanisms to cope with fluctuating light.

Photosynthetic control at the Cyt *b*$_6$*f* complex might be more important in regulation of the electron flow in FL, especially in terms of protection of PSI. In contrast to the down-regulation of PSII in the antenna system or the PSII reaction centers detected as NPQ, photosynthetic control at the Cyt *b*$_6$*f* complex is considered as a regulation mechanism that tunes the electron flow rate to the Cyt *b*$_6$*f* complex onwards (West and Wiskich 1968; Rumberg and Siggel 1969; Hall et al. 1971) depending on the phosphate potential in chloroplasts (Kramer et al. 1999; Tikhonov 2013). When ATP synthesis occurs intensively, protons are excreted from the lumen to stroma through the H^+-ATP synthase, preventing excessive acidification of the lumen. The moderately acidic pH allows high rate of electron transfer to PSI. However, when ADP and Pi are in shortage, production of ATP is suppressed, lumen pH decreases and thereby the electron transport is decelerated (Takizawa et al. 2008; Kiirats et al. 2009). In the proton-coupled electron transport in the Cyt *b*$_6$*f* complex, the oxidation of plastoquinol (PQH_2) at the Q_0 site is the rate-limiting step. Involvement of the H^+-ATP synthase in the photosynthetic control is also suggested (Rott et al. 2011). Thus, the light-induced acidification of the lumen is the main factor of the feedback control of the linear electron transport in chloroplasts.

In addition to the LEF system, plants have some photosynthetic alternative electron flows in the chloroplast. There are two cyclic electron flow around PSI (CEF-PSI) systems (Shikanai 2007): the NADH dehydrogenase-

like complex-dependent pathway (NDH-mediated CEF, Burrows et al. 1998; Shikanai et al. 1998; Peng et al. 2011; Yamamoto et al. 2011) and the PGR5-mediated pathway (PGR5-mediated CEF, Munekage et al. 2002, 2004; DalCorso et al. 2008; Hertle et al. 2013). The PGR5-mediated CEF involves plastoquinone (PQ), the Cyt *b*$_6$*f* complex, plastocyanin (PC), PSI, ferredoxin (Fd) and ferredoxin-plastoquinone reductase (FQR). PGR5 and PGRL1 were identified as essential components of the PGR5-mediated CEF (Munekage et al. 2002; Hertle et al. 2013). The CEF-PSI would contribute to producing additional ATP but not NADPH. Another function of CEF-PSI is enhancement of the NPQ, through generating the electrochemical potential difference of H^+ across the thylakoid membrane (Munekage et al 2002).

The water-water cycle (WWC), also called the Asada cycle (Asada 1999) or the Mehler-ascorbate peroxidase (MAP) pathway (Schreiber et al. 1995), is the pseudo-cyclic electron flow from water via PSII, Cyt *b*$_6$*f*, and PSI to molecular oxygen. This cycle leads to formation of reactive oxygen species (ROS), such as O_2^- and H_2O_2 (Asada 1999). Superoxide dismutase and ascorbate peroxidase scavenge O_2^- and H_2O_2. Namely, electrons in the WWC are transferred from water to H_2O_2 to form water. Because of these reactions the WWC acts as a large electron sink (Asada 2000). Therefore, the WWC is also considered to play roles in dissipation of excess light energy (Osmond and Grace 1995; Osmond et al. 1997; Asada 1999, 2000; Foyer and Noctor 2000; Miyake 2010). The WWC also generates the ΔpH across the thylakoid membrane, which enhances NPQ. The CEF-PSI and WWC are argued to protect plants from damages that occur due to the over-reduction of the

thylakoids under stress conditions (Miyake 2010).

The cyclic electron transport involving PGR5 protein has been proposed to protect PSI from the fluctuating growth light in *A. thaliana* (Suorsa et al. 2012). The Arabidopsis *pgr5* mutant deficient in PGR5 showed a lethal phenotype in fluctuating growth light (Tikkanen et al. 2010). The excitation energy balance mediated by the LHCII phosphorylation, which differs from the traditional state transitions, is also recently suggested to be a key factor for acclimation to the fluctuating light because this contributes to maintenance of the balanced activity of PSI (Grieco et al. 2012). As described clearly in Chapter 2, the photosynthetic alternative electron flows, especially the PGR5-mediated CEF, are essential for protection of PSI from FLP.

Most of the studies of photoinhibition are focusing on PSII, because PSII is highly sensitive to light but not PSI, and because mechamisms of both photodamage and repair of PSII are attractive. However, the knowledge has been mainly obtained in the laboratory using continuous light as the photoinhibitory light. How fluctuating light influences PSII photoinhibition remains largely unknown (Sarvikas et al. 2010). If PSI photoinhibition accelerates PSII photoinhibition, photoinhibition in continuous light may differ from that in fluctuating light. Photoinhibition of both PSII and PSI should be evaluated simultaneously in fluctuating light to understand photoinhibition in the field.

In this study, I first examined the effects of fluctuating light on photoinhibition of both PSII and PSI, using simultaneous chlorophyll

fluorescence and P700 measurements. I adopted three fluctuating light regimes including the moderate fluctuating light (FL-240/30) that altered between HL phase at 240 μmol photons m^{-2} s^{-1} for 2 min and LL phase at 30 μmol photons m^{-2} s^{-1} for 2 min, high fluctuating light (FL-1200/30) that altered between HL phase at 1200 μmol photons m^{-2} s^{-1} for 2 min and LL phase at 30 μmol photons m^{-2} s^{-1} for 2 min and the highest fluctuating light (FL-1200/240) that altered between HL phase at 1200 μmol photons m^{-2} s^{-1} for 2 min and LL phase at 240 μmol photons m^{-2} s^{-1} for 2 min. Next, I evaluated how the differences in the mode of light fluctuation influence responses of the photosynthetic electron transport system. I measured PSII and PSI parameters in response to the different modes of the short-term fluctuating light for 162 min. Finally, the effects of fluctuating light on regulation of the photosynthetic electron transport system were evaluated. I examined roles of non-photochemical energy dissipation system in the PSII antenna system, and the photosynthetic alternative electron flows in response to the fluctuating light using the *npq4* mutant and the over-expression line of PGR5.

3.2. Materials and methods

Plant materials

Arabidopsis thaliana wild-type (Columbia-0) plants were pot-grown in a growth cabinet with white fluorescent light at a photosynthetically active photon flux density (PPFD) of 135 μmol photons m^{-2} s^{-1} for 8-h photoperiod at room temperature of 23℃ and relative humidity of 60%. Plants were irrigated two to three times weekly and were fertilized with Hyponex 6-10-5 solution (Hyponex Japan, Osaka, Japan) diluted to the 1: 500 strength every irrigation from two weeks after germination. Mature rosette leaves from 7- to 9-week-old plants were detached, and these detached leaves with their petioles in distilled water were used in all experiments. I detected no differences in the obtained results between in intact leaves and in cut leaves.

In this study, an *A. thaliana* mutant, *npq4* and an over-expression line of PGR5, were also used. For the detail of *npq4* and the over-expression line of PGR5, see Li et al (2000) and Okegawa et al. (2007), respectively. The growth conditions of these plants were the same as those for the wild type.

Fluctuating- and continuous- light treatments

In this study, I defined the fluctuating light as the alternating between high light (HL) phase and low light (LL) phase with a constant time intervals. Three fluctuating light (FL) regimes and continuous light (CL) at contrasting PPFD levels were used (Table 1). Three fluctuating light regimes included the

moderate fluctuating light (FL-240/30) that altered between HL phase at 240 μmol photons m^{-2} s^{-1} for 2 min and LL phase at 30 μmol photons m^{-2} s^{-1} for 2 min, high fluctuating light (FL-1200/30) that altered between HL phase at 1200 μmol photons m^{-2} s^{-1} for 2 min and LL phase at 30 μmol photons m^{-2} s^{-1} for 2 min and the highest fluctuating light (FL-1200/240) that altered between HL phase at 1200 μmol photons m^{-2} s^{-1} for 2 min and LL phase at 240 μmol photons m^{-2} s^{-1} for 2 min. The continuous moderate- and high-light were at PPFDs of 240 (CL-240) and 1200 (CL-1200) μmol photons m^{-2} s^{-1}, respectively. Red light-emitting diodes (LEDs) with a wavelength peak at 635 nm were used as the light source. The LEDs is installed in the central unit of a DUAL-PAM-100. PPFD level was controlled by a program running on a personal computer (PAM program). This enabled very quick changes in PPFD.

Chlorophyll fluorescence and 830 nm absorbance change measurements

Chlorophyll fluorescence and absorption changes at 830 nm were measured simultaneously using the Dual-PAM-100 (chlorophyll fluorescence and P700 absorption analyzer equipped with a P700-dual wavelength-emitter at 830 and 875 nm, Walz, Effeltrich, Germany) with the cut leaf in room air. Saturation pulses (SPs) from red LEDs (> 6000 μmol photons m^{-2} s^{-1}, 250 ms duration) were applied to determine the maximum chlorophyll fluorescence with closed PSII centers in the dark (F_m) and in the actinic light (F_m'). Maximum photochemical quantum yield of PSII (F_v/F_m) and effective

quantum yield of PSII (Y(II)) were calculated as $(F_m - F_0)/F_m$ and $(F_m' - F_s')/F_m'$ (Genty et al. 1989), respectively, where F_s' is the steady-state chlorophyll fluorescence level in the actinic light from red LEDs with wavelength peak at 635 nm. At this wavelength, chloroplast avoidance movement does not occur and has no effects on assessment of non-photochemical quenching components (Cazzaniga et al. 2013). Two other PSII quantum yields, Y(NPQ) and Y(NO) (Genty et al. 1996; Kramer et al. 2004a), which represent the regulated and non-regulated energy dissipation at PSII centers, respectively, and add up to unity with the photochemical quantum yield (i.e. Y(II) + Y(NPQ) + Y(NO) = 1), were also used. Y(NPQ) and Y(NO) were calculated as $F_s'/F_m' - F_s'/F_m$ and F_s'/F_m, respectively (Hendrickson et al. 2004; Klughammer and Schreiber 2008a). The coefficient of non-photochemical quenching, qN, was calculated as $(F_m - F_m')/(F_m - F_0')$ (Schreiber et al. 1986). The coefficient of photochemical quenching, qP and qL, a measure of the fraction of open PSII reaction centers, based on the "puddle model" and "lake model" of PSII antenna pigment organization, respectively, were calculated as $(F_m' - F_s')/(F_m' - F_0)$ (Schreiber et al. 1986) and $(F_m' - F_s')/(F_m' - F_0') \cdot F_0'/F_s'$ (Kramer et al. 2004b), respectively. F_0-quenching, q_0, was calculated as $(F_0 - F_0')/F_0$, which expresses the quenching coefficient of the increment of heat dissipation in the light (Bilger and Schreiber 1986; Bukhov et al. 2001). Excess parameter, Y(EX), was calculated as $(F_s - F_0')/F_m'$, which expresses the fraction of energy that is neither used for photochemistry nor dissipated as heat (Demmig-Adams et al. 1996). F_0' is minimal fluorescence yield in the actinic light and was estimated using the approximation of

Oxborough and Baker (1997) as $F_0/(F_v/F_m + F_0/F_m')$. Using Y(EX), the rate of excess energy production was estimated from Y(EX) × 0.5 × 0.84 × PPFD . 0.5 is the share of absorbed light energy allocated to PSII and 0.84 is leaf absorptance.

With the Dual-PAM-100, $P700^+$ was monitored as the absorption difference between 830 and 875 nm in a transmission mode. In analogy to chlorophyll fluorescence yields, the quantum yields of PSI was determined using the saturation pulse method (Klughammer and Schreiber 1994; Klughammer and Schreiber 2008b). Maximum level of P700 signal (P700 fully oxidized) in the dark, called $P_{m,}$ was determined by application of a SP in the presence of far-red light at the wavelength of 720 nm. This parameter is also written as ΔA_{max}, which means maximal absorption change. The zero P700 signal, P_0, was determined when complete reduction of P700 was induced after the SP in the absence of far-red light. P_m' is the maximal P700 signal in the presence of actinic light induced by the SP. The photochemical quantum yield of PSI, Y(I), was calculated from the complementary PSI quantum yields of non-photochemical energy dissipation, Y(ND) and Y(NA), respectively: $Y(I) = 1 – Y(ND) – Y(NA)$. Y(ND) corresponds to the fraction of P700 that is already oxidized by actinic light, and Y(NA) corresponds to the fraction of P700 that cannot be oxidized by a SP to the all P700. These calculations were made according to Klughammer and Schreiber (2008b). I paid attention to the photoinhibitory effect on PSI by SP because consecutive short-saturating flashes applied in the dark cause photodamage to PSI (Sejima et al. 2014). To oxidize the inter-system electron carriers, far-red light

was applied from 100 ms before the start of the SP to its cessation. My preliminary checks showed that the SP of 250 ms duration was enough to induce maximal $P700^+$ oxidation level and to obtain the complete reduction level of P700 after the SP. Photodamage by the SP did not occur in the presence of actinic light.

Measurement of the P515 (ECS)

The proton motive force (pmf) was assessed by measuring the electrochromic pigment absorbance shift (ECS) via dual-wavelength (550-515 nm) transmittance changes (P515) with the DUAL-PAM-100 with a P515/535 module. The P515/535 module consists of an emitter unit DUAL-EP515 and a detector unit DUAL-DP515. The P515 signal was the transmittance at 550 nm – that at 515 nm difference signal, in which the 550 nm was chosen as the reference wavelength in order to minimize any contribution of absorbance change by zeaxanthin. Valuable information on the thylakoid membrane potential ($\Delta\Psi$) and proton gradient (ΔpH) components of the pmf are obtained by analyzing dark-interval relaxation kinetics of the P515 signal (DIRK-analysis, Cruz et al. 2001; Avenson et al. 2004). For determination of the rate of proton efflux via the H^+-ATPase, the DIRK-analysis has been used (Sacksteder et al. 2000; Kanazawa and Kramer 2002; Kramer et al. 2003; Cruz et al. 2005). I applied the DIRK-analysis and measured the continuous changes in light-driven charge fluxes in photosynthetic electron transport chain, which technique is involved in 1:1 light:dark modulation of actinic light

with alternation from several to several tens of ms (Schreiber and Klughammer 2008; Klughammer et al. 2013). In this study, the actinic light was modulated 20 ms on/off periods. The resulting "P515 flux" signal means changes in the initial slope of the dark-interval P515 decay, which provides "continuous" information on the rate of proton efflux via the H^+-ATPase. The changes in P515 flux signal were measured during the moderate-FL for 162 min. Time integrated PPFD was set at 240 μmol photons m^{-2} s^{-1} for the HL phases and 30 μmol photons m^{-2} s^{-1} for the LL phases, which means actual PPFDs for 20 ms on-periods in high- and low-actinic light phases were at 480 and 60 μmol photons m^{-2} s^{-1}, respectively. For details, see Klughammer et al. (2013).

Determination of rate constants

Based on a theory that photodamage to PSII depends on the fraction of excess light energy (excess energy theory), I determined the rate constant of photodamage of PSII ($k_{pi,PSII}$) using lincomycin, an inhibitor of chloroplast-encoded protein synthesis. Petiole of cut leaves were soaked in 1mM lincomycin solution and kept in the dark for 16 h at 23℃. Assuming that the rates of photodamage and recovery ($k_{rec,PSII}$) are proportional to the concentrations of non-functional and functional PSII, respectively, the fraction of active PSII (a) is expressed as:

$$a = \{k_{rec} + k_{pi} \cdot \exp[-(k_{pi} + k_{rec}) \cdot t]\} \cdot (k_{pi} + k_{rec})^{-1}$$

(see Kok 1956; Tyystjärvi et al. 1992; Wünschmann and Brand 1992), where a is the ratio of F_v/F_m after light-treatments in the absence of lincomycin, relative to the initial value, and t is illumination time (min). When k_{rec} is zero, in which lincomycin is added, the fraction of inactive PSII (b) is expressed as:

$$b = \exp(-k_{pi} \times t)$$

, where b is the ratio of F_v/F_m after light-treatment for t min in the presence of lincomycin, relative to the initial value. The details are described in Kato et al. (2002).

3.3. Results

Light responses of the steady-state PSII and PSI parameters

Light responses of the steady-state PSII and PSI parameters were analyzed (Fig. 1). The fluorescence parameters were the quantum yields of the PSII photochemistry, Y(II), non-regulated energy dissipation, Y(NO), and regulated energy dissipation, Y(NPQ) (triangle). For energy captured by PSI pigments, the quantum yields of the PSI photochemistry, Y(I), non-photochemical energy dissipation due to the donor-side limitation of PSI, Y(ND), and non-photochemical energy dissipation due to the acceptor-side limitation of PSI, Y(NA), were measured. Y(II) and Y(I) decreased with the increment in PPFD, while, at PPFDs from 20 to 45 μmol m^{-2} s^{-1}, both of these yields were slightly higher than those at lower PPFDs. With the increment in PPFD, Y(NPQ) marked increased, especially above at PPFD of 250 μmol photons m^{-2} s^{-1}, while Y(NO) increased only slightly. Y(NA) attained a maximum at 250 μmol photons m^{-2} s^{-1} and was greater than Y(ND) at PPFDs above 390 μmol photons m^{-2} s^{-1}. Above this level, Y(ND) increased considerably with complementing with the decrement in Y(I).

Effects of fluctuating light on photoinhibition of PSII and PSI in the absence of lincomycin

The effects of fluctuating- and continuous- light on photoinhibition of PSII and PSI were examined in the col-0 plants leaves in the absence of lincomycin (Fig. 2). Photoinhibition of PSII and that of PSI were assessed as the reduction of F_v/F_m and that of ΔA_{max}, respectively. PSII and PSI showed no photoinhibition in the moderate continuous light at 240 μmol photons m^{-2} s^{-1} (CL-240) for 162 min (Fig. 2A and B). At 1200 μmol photons m^{-2} s^{-1} (CL-1200), PSI was not photoinhibited and PSII slightly photoinhibited for 162 min (Fig. 2C and D). In contrast, exposure to the fluctuating light, FL-240/30 or FL-1200/30FL-240/30 caused marked photoinhibition of PSI (Fig.2B and D). The extents of PSI photoinhibition in the FL increased with the illumination time, and ΔA_{max} after the FL for 162 min decreased to about 70% of the initial level in both FL-240/30 and FL-1200/30, while the extents of PSII photoinhibition in these FL regimes were similar to that in the CL-240 and CL-1200, respectively (Fig. 2A and C). When the FL-1200/240 was applied, the extent of PSI photoinhibition was similar to those in the FL-240/30 and FL-1200/30 (Fig. 2F).

Effects of fluctuating light on photoinactivation of PSII and PSI in the presence of lincomycin

In the presence of lincomycin, which inhibits the synthesis of D1 protein in PSII, the reduction of F_v/F_m with time was greater (Fig. 3A, C and E) than in untreated leaves (Fig. 2A, C and E). Since the de novo synthesis of D1 protein

was inhibited by lincomycin, the reduction of F_v/F_m reflected photodamage. The extent of PSII photodamage was greater in FL-1200/30 and FL-1200/240 than in FL-240/30-240/30, and greater in the CL-1200 than in in the CL-240. The extents of PSII photoinhibition were similar between the FL-240/30-240/30 and CL-240, and among the FL-1200/30, FL-1200/240 and CL-1200, respectively (Fig. 3A, C and E). On the other hand, PSI photoinhibition was considerably smaller in the lincomycin-treated leaves (Fig. 3B, D and F) than in the untreated leaves in FL (Fig. 2B, D and F).

Effects of PSI photoinhibition on the far-red light dependency of P700 signal

To confirm the data of PSI photoinhibition evaluated by the saturation pulse method, I measured the extent of far-red light induced P700 oxidation. Fig. 4 shows changes in P700 signals in response to far-red light illumination before and after the FL-240/30 treatment. In the dark-adapted leaf before the FL-240/30 treatment, P700 signal increased rapidly upon far-red illumination and reached a plateau via a dip. In contrast, in the same leaf after the FL-240/30 treatment for 162 min, the steady-state value of the P700 signal under the far-red was lower than that before the FL treatment. These indicate that PSI was photoinhibited by the FL treatments and that the evaluation of P700 activity by the saturation pulse method was valid.

Is photodamage of PSII by excess energy accelerated in fluctuating light?

From Fig.3, I calculated rate constants of photodamage of PSII ($k_{pi,PSII}$), using the first-order equation (Kok 1956) (Table 2). The $k_{pi,PSII}$ in the FL-240/30 was similar to that in the CL-240, while the $k_{pi,PSII}$ in the FL-1200/30 and the FL-1200/240 were lower than that in the CL-1200 by about 30%. The $k_{pi,PSII}$ in FL-1200/30 was similar to that in FL-1200/240. On the other hand, the rate constant for the recovery of PSII from photodamage ($k_{rec,PSII}$) was largest in FL-240/30-240/30 and second largest in CL-240. The $k_{rec,PSII}$ in CL-1200was largerer than that in FL-1200/30. The $k_{rec,PSII}$ was smallest in FL-1200/240.

One of the hypotheses for the mechanism of PSII phodamage is that the fraction of energy that is neither used for photochemistry in PSII nor dissipated as heat, called Y(EX) in this study, is responsible for the photodamage to PSII (Demmig-Adams et al. 1996). Indeed, the absolute values of the excess energy calculated as Y(EX) × PPFD × 0.84 ×0.5 has been shown to be proportional to the rate constant of PSII photodamage (Kato et al. 2003; Kornyeyev et al. 2003). I examined the relationships between the rate constant of PSII photodamage ($k_{pi,PSII}$) and the total amount of excess energy during the FL and CL for 162 min (Fig. 5). There were linear relationships between FL-240/30 and CL-240, and among the FL-1200/30, FL-1200/240 and CL-1200.

Responses of PSII and PSI parameters in response to the FL-1200/30 and FL-240/30

Changes in the PSII (Fig. 6) and PSI parameters (Fig. 7) were measured in the FL-1200/30 and FL-240/30 for 162 min. Fig. 6 shows the data for 8 min of five parts during the treatment for 162 min. The leaves were dark-adapted for 30 min before the light treatments. In the leaves in FL-1200/30, the electron transport rate through PSII, ETR(II), kept pace with the changes in PPFD: namely ETR(II) in HL phases did not change after attaining the steady value except for first several cycles (Fig. 6A). Similarly, Y(NPQ) in HL phases showed the steady value over the treatments for 162 min, from the second cycle (Fig. 6B). Y(NO) showed constant values regardless of HL- and LL-phases, although Y(NO) increased rapidly upon a transition from dark to the first HL (Fig. 6C). A measure of the fraction of reduced PQ, 1－qP, in HL phases kept high values except for the first several cycles, and 1－qP in LL phases increased slightly stepwise with the cycles (Fig. 6D). On the other hand, in the leaves in FL-240/30, ETR(II) in HL phases decreased stepwise with the cycles after several ten minutes (Fig. 6A). In contrast to Y(NPQ) in the FL-1200/30, Y(NPQ) in HL phases in the FL-240/30 showed the highest values at the first measurement for each HL phase, followed by the decreases till the end of the HL phases (Fig. 6B). The degree of the decrease during the HL phase became greater with the cycle. Moreover, Y(NPQ) in LL phases was much smaller in the FL-240/30 than in FL-1200/30. Y(NO) in FL-240/30

increased gradually with the cycles (Fig. 6C). In the first several ten minutes, Y(NO) at the first measuring point of each HL phase increased rapidly, but for the rest of the cycles, no such spikes of Y(NO) were observed upon the onset of HL. Although 1−qP in LL phases in the FL-240/30 was lower than that in the FL, it increased stepwise with the cycles, reaching similar values to that in the high-FL (Fig. 6D). Excess energy in HL phases increased slightly with the cycles.

PSI parameters were also measured simultaneously (Fig. 7). In analogy with the results of ETR(II), ETR(I), shown as closed symbols, kept pace with the changes in PPFD during the high-FL, while in FL-240/30, shown as open symbols, ETR(I) in HL phases decreased stepwise with the cycles (Fig. 7A). Y(ND) in HL phases was considerably higher in the high-FL. In both high- and FL-240/30, Y(ND) decreased with the cycles (Fig. 7B), resulting in the increases in Y(NA). This increases in Y(NA) were larger in the FL-240/30 than in FL-1200/30 (Fig. 7C).

Are there differences in the quenching mechanism between fluctuating- and continuous-light?

The heat dissipation is proposed to occur in the light-harvesting antenna of PSII (Horton et al. 2000) or in the reaction center of PSII (Weis and Berry 1987). The non-photochemical parameter, qN, is used frequently as a measure of the dissipation of excess excitation energy (Schreiber et al. 1986), whereas

F_0-quenching, q_0, expresses the increment of heat dissipation in the light (Bilger and Schreiber 1986; Bukhov et al. 2001). According to Stefanov and Terashima (2008), I tested whether there are differences in fluorescence origin among various light treatments by analyzing the relationships between q_0 and qN (Fig. 8). The relationships between q_0 and qN did not differ by the modes of light treatment, indicating that there were no differences in the quenching mechanisms among light treatments. It was obvious that a variation range of qN to the light treatments was more widespread in the fluctuating light than in the continuous light because of the presence of LL phases.

Role of qE-quenching in protecting PSII and PSI from photoinhibition in short-term fluctuating light

NPQ contributes to down-regulation of the photosynthetic electron transport in the antenna of PSII. qE-quenching is supposed to be main process of NPQ. Previous reports have indicated that mutants impaired in qE-quenching, *npq1* and *npq4*, are sensitive to high light illumination (Niyogi et al. 1998; Li et al. 2002), and physiological importance of qE-quenching in the field (Külheim et al. 2002). To evaluate a role of qE-quenching in protection from photoinhibition in short-term fluctuating light, I performed the same measurements as Fig. 2 using the *npq4* mutant, deficient in PsbS protein (Li et al. 2000). The plants were grown in continuous light at 135 μmol photons

m^{-2} s^{-1} (Fig. 9). Fig. 9 shows the reduction of F_v/F_m and ΔA_{max} in the FL-240/30 (A and C) and FL-1200/30 (B and D) in the absence of lincomycin. Sensitivity of the *npq4* leaves to the FL-1200/30 was somewhat greater than that of col-0, although in FL-240/30, differences were not detected. For photoinhibition of PSI, *npq4* were more resistant, although the differences were not marked.

Involvement in the NDH-mediated CEF

To examine whether NDH-mediated CEF functions in the fluctuating- and continuous-light for 162 min, I measured a transient post-illumination increase in chlorophyll fluorescence (Fig. 10), which is manifested by a result of NDH-dependent reduction of the PQ pool in the dark (Shikanai et al. 1998). The transient increase in chlorophyll fluorescence was detected in leaves exposed to the CL-1200 and FL-1200/30 (Fig. 10B and D), implying that NDH-mediated CEF activated under these light conditions. The amplitude of post-illumination rise in chlorophyll fluorescence was greater in leaves in the FL-1200/30 than those in the HL-1200. However, the transient rise in chlorophyll fluorescence was not observed in leaves exposed to the CL-240 and FL-240/30 (Fig. 10A and C). The absence of the increase in chlorophyll fluorescence is consistent with the phenotypes of *crr* mutants specifically defective in NDH (Shikanai et al. 1998; Shimizu et al. 2008; Peng et al. 2009; Yamamoto et al. 2011).

Role of PGR5 protein in protecting PSI from photoinhibition in short-term fluctuating light

Some previous reports suggested that PGR5 protein protects PSI from photodamage under fluctuating growth light (Suorsa et al. 2012). Furthermore, in Chapter 2, I showed that the PGR5-medited CEF is essential for regulation of the photosynthetic electron transport system in response to FL. I examined whether the over-expression of PGR5 enhance the tolerance to the FL. The over-expression line of PGR5 was grown in the continuous light at 135 μmol photons m^{-2} s^{-1} and the same photoinhibition measurements as Fig.2 were performed (Fig. 11). Compared with the wild type, ΔA_{max} in the over-expression line decreased only slightly after the FL-240/30 and FL-1200/30 for 162 min. PSII photoinhibition was not detected in *pgr5OX*.

Rapid P515 relaxation kinetics induced by single-turnover saturating flash

I examined whether H^+-ATPase in the thylakoid membrane was inactivated during the FL treatments. Fig. 12 shows P515 absorbance changes induced by a saturation single turnover flash in the same leaf. Fig.12A shows the response of the leaf that had been kept in the dark for 30 min. A rapid rise of

P515 signal was followed by a distinct slow rise phase, with the overall response peaking at about 15 ms, and by a slow decline phase. In Fig. 12B, the response of the same leaf after the treatment with the CL-240 for 162 min. After the treatment, the leaf was kept in the dark for 5 min and P515 changes were measured. P515 increased rapidly by the flash and decayed very rapidly without the slow rise phase. For the same leaf illuminated with the FL-240/30 for 162 min, followed by the dark treatment for 30 min, the kinetics of the flash-induced P515 change was similar to that of the leaf kept in the dark for 30 min shown as Fig. 12A (Fig. 12C).

Measurements of the P515 flux signal in response to the FL-240/30

To understand the relationships between PSII photoinhbition and PSI photoinhibition in more detail, I needed more information of "charge flux" as well as of PSII and PSI parameters. Recently, Klughammer et al. (2013) has developed a new technique of continuous measurement of charge flux. This technique is based on extensive studies by Joliot, Kramer and co-workers on dark-interval relaxation kinetics (DIRK) of P515, which contain information not only on the pmf and its partitioning into its ΔpH and $\Delta\Psi$ components (Cruz et al. 2001; Avenson et al. 2004), but also on the light-driven charge flux (Sacksteder et al. 2000; Kanazawa and Kramer 2002; Kramer et al. 2003; Cruz et al. 2005). A reliable continuous measure of light-driven charge fluxes in photosynthetic electron transport system was allowed by modulating

actinic light into 1:1 light:dark with alternating 20 ms pulses. The resulting "P515 flux" signal means the changes in the initial slope of the dark-interval P515 decay, which provides information on the rate of proton efflux via the ATPase. I measured P515 flux signal during the FL-240/30 for 162 min with the actinic light modulated 20 ms on/off periods (Fig. 13). Time integrated PPFD was set at 240 μmol photons m^{-2} s^{-1} for the HL phases and 30 μmol photons m^{-2} s^{-1} for the LL phases, which means actual PPFDs for 20 ms on-periods in high- and low-actinic light phases were at 480 and 60 μmol photons m^{-2} s^{-1}, respectively. When leaves were exposed to HL phases, P515 flux signal showed a spike, followed by subsequent increases. Except for the first cycle, the signal in HL phases reached the maximum level within first 30 seconds from the onset of the HL phases, and subsequently decreased slowly. This kinetics continued to the last cycle, and the average of the signals in HL phases for 2 min did not decrease. The signal in LL phases gradually decreased for the first 30 sec from the phase change and subsequently reached the steady-state. This steady-state means that a flux equilibrium was established between formation of the $\Delta\psi$ via charge separation at the reaction centers and relaxation of $\Delta\psi$ caused by H^+ efflux via the H^+-ATPase.

3.4. Discussion

Fluctuating light photoinhibition (FLP)

The present data, such as those in Figs. 2 and 4, clearly showed that the "light fluctuation" is a potent stress factor. This is consistent with that of Chapter 2. Even when PPFD in HL phase was moderate, at 240 μmol photons m^{-2} s^{-1}, leaves of the wild type plants grown in the CL were photoinhibited by the FL. The main target of fluctuating light photoinhibition (FLP) is PSI because the CL used in this study did not cause PSI photoinhibition. Interestingly, the degree of PSI photoinhibition did not differ irrespective of the FL-240/30, FL-1200/30 and FL-1200/240. In the FL-240/30, insufficient activation of the photoprotective processes in chloroplasts led to marked photoinhibition of PSI for these PPFD levels. Protection of PSI from the FLP needed to involve in the non-photochemical energy dissipation in PSII antenna system, the down-regulation of the electron flow at the Cyt *b*$_6$*f* complex and the cyclic electron flows around PSI (CEF-PSI), especially the PGR5-mediated CEF. These resulted in apparently similar PSI photoinhibition irrespective of the PPFD levels. It should be also stressed that PSI photoinhibition did not accelerate PSII photoinhibition because alternative electron flows compensated for the electron flow through PSI, and consequently keep the photosynthetic electron transport through PSII to a sufficient level.

Effects of different modes of the FL on the photosynthetic electron transport system

Although the PPFD in HL phase and total fluence were lower in FL-240/30 than in FL-1200/30 or in Fl-1200/240, the extents of PSI photoinhibition were similar among these FL treatments. Then, following questions arise. Why were the extents of PSI photoinhbition similar regardless of the large differences in fluence of the FL? If excess energy is not responsible for the damage, what does accelerate PSI photoinhibition in FL? In the FL-1200/30, ETR(II) and ETR(I) in HL phases were constant and did not decreased with the cycles (Fig. 6). Y(NPQ) in HL phases was saturated in the early cycles of the FL, indicating that NPQ was activated sufficiently. The higher values of Y(NPQ) in LL phases than those in the FL-240/30 would enable the rapid increase in Y(NPQ) to the sudden increase in PPFD. Similarly, Y(ND) in HL phases was higher than that in the FL-240/30. On the other hand, in response to FL-240/30, ETR(II) and ETR(I) in HL phases decreased stepwise with the cycle (Fig. 6 and 7). Y(NPQ) values in both HL and LL phases were lower and Y(ND) was markedly lower than those in FL-1200/30 or FL-1200/240. Excess energy increased with the cycles. These results suggest that the non-photochemical energy dissipation in PSII was insufficient to down-regulate PSII and, thus, the donor-side limitation of PSI was low. Consequently, PSI was photodamaged to a considerable extent.

In summary, In FL, Y(NPQ) has to be maintained at relatively high level even in LL phases, which would enable the quick further increase in Y(NPQ)

in HL phases. The same would apply to the donor-side limitation of PSI. Then, the photosynthetic electron transport system can quickly respond to the sudden increase in PPFD, leading to protection of PSI from photodamage. In FL-1200/30, tight regulation by these systems would suppress the electron flow to PSI and thereby that to oxygen. This would avoid photo-oxidative damage to PSI by ROS as described in Chapter 2. In contrast, in FL-240/30, incomplete suppression of the electron flow to oxygen would result in PSI photoinhibition. In addition, Y(NA) levels, the acceptor-side limitation of PSI, were higher towards the end of the FL treatment in FL-240/30 than in FL-1200/30 (Fig. 7C), supports these interpretations.

Roles of non-photochemical quenching in regulation of the photosynthetic electron transport system in short-term FL

Physiological importance of qE-quenching for plant performance has been suggested by the observation using *A. thaliana* mutants, *npq1* and *npq4*, with an impaired xanthophyll cycle and deficient in the PsbS protein, respectively (Külheim et al. 2002). I examined the roles of qE-quenching in regulation of the photosynthetic electron transport system in response to the short-term FL. The degree of PSI photoinhibition in *npq4* in the FL-1200/30 was similar to that in wild type, but that in the FL-240/30 was smaller than that in wild type (Fig. 9). In contrast, the degree of PSII photoinhibition in *npq4* in the FL-1200/30 was slightly larger than that in wild type. These results might

indicate that roles of qE-quenching in responses to the short-term FL are not essential for protecting both PSII and PSI from FLP. qE-quenching is reversible and flexible (Bianchi et al. 2010; Ruban et al. 2012), which is triggered by the light-induced acidification of the thylakoid lumen. Acidification of the lumen induces the qE-quenching through protonation of PsbS protein and activation of the xanthophyll cycle. Collaboration of these two pH-induced processes allows the accomplishment of a maximal performance of qE-quenching (Muller et al. 2001; Ruban et al. 2012). Because the *npq4* mutant is deficient in PsbS protein (Li et al. 2000), xanthophyll cycle-dependent qE-quenching and other quenching processes may compensate for a function of PsbS-dependent qE-quenching. According to a paper that the PsbS protein plays a role of a kinetic modulator of the energy dissipation process in the PSII antenna system, *npq4* possesses alternative photoprotective energy dissipation, which works on a slower timescale for 1 h (Johnson and Ruban 2010). Other explanation is that npq4 may have large share of light energy allocated PSI. Consequently, increased CEF-PSI may protect PSI from the FLP. Further studies have to be required to evaluate a role of the PsbS-dependent NPQ or PsbS protein in response to the light fluctuations.

Roles of photosynthetic control at the Cyt *b*$_6$*f* complex in protection of PSI from the FLP

For light responses curves, the donor-side limitation of PSI shown as Y(ND) increased with the increases in PPFD (Fig. 1). Similarly, Y(ND) were large in HL phases in the FL, especially FL-1200/30 (Fig. 6). Photosynthetic control at the Cyt *b*$_6$*f* complex and/or PSII photoinhibition are considered to contribute to Y(ND) because these processes down-regulate the electron flow to PSI. In this study, PSII photoinhibition by the FL was slight (Fig. 2). In addition, Y(ND) in LL phases in the FL decreased with the cycles. Thus, I considered that the high Y(ND) in the FL was responsible for the down-regulation by Photosynthetic control at the Cyt *b*$_6$*f* complex.

Photosynthetic control at the Cyt *b*$_6$*f* complex might be more important in regulation of the electron flow in FL, especially in terms of protection of PSI. In contrast to the down-regulation of PSII in the antenna system or the PSII reaction centers detected as NPQ, photosynthetic control at the Cyt *b*$_6$*f* complex is considered as a regulation mechanism that tunes electron flow rate to the Cyt *b*$_6$*f* complex onwards (West and Wiskich 1968; Rumberg and Siggel 1969; Hall et al. 1971). My data indicate that photosynthetic control at the Cyt *b*$_6$*f* complex, shown as Y(ND), protected PSI in the FL, especially in FL-1200/30, because Y(I) did not decrease in the FL1200/30 (Fig. 7). The increases in Y(NA), the acceptor-side limitation of PSI, were smaller in the FL-1200/30 than in FL-240/30. This suggests that photosynthetic control avoid the electron flow to oxygen molecules. However, it is also possible that photoprotective mechanism due to the photosynthetic control could be too slow to be active enough upon the extremely rapid increase in PPFD, because the light-induced acidification of the lumen has

a lag.

The *pgr5* (*proton gradient regulation-5*) mutant, isolated on the basis of its high chlorophyll fluorescence at high PPFD (Munekage et al. 2002), has been proposed to be deficient in the photosynthetic control at the Cyt b_6f complex (Avenson et al. 2005; Suorsa et al. 2012; Fisher and Kramer 2014) and in the CEF-PSI (Munekage et al. 2002). Although there have been controversial views concerning the CEF-PSI capacity of *pgr5*, I confirmed in Chapter 2 that *pgr5* has a capacity for the CEF-PSI. However, the actual activity of the CEF-PSI in *pgr5* is low. When grown in the FL, *pgr5* showed a lethal phenotype (Tikkanen et al. 2010; Suorsa et al. 2012). Moreover, *pgr5* grown in the CL showed marked PSI photoinhibition even after short-term exposure to the FL-240/30 for 40 min as has been shown in Chapter 2. The donor-side limitation of PSI in *pgr5* was almost zero regardless of fluctuating- and continuous-light. However, at low oxygen concentrations, the CEF-PSI was enhanced, resulting in no PSI photoinhibition in the FL. CEF-PSI, especially PGR5-mediated CEF, may be most effective mechanism to protect PSI.

Roles of the PSII photodamage-repair cycle in protection of PSI from the FLP

Besides energy dissipation in the PSII antenna system and the photosynthetic control, photodamage of PSII has been also regarded as a

mechanism that protects PSI (Sonoike 2011; Tikkanen et al. 2013). Electrons from PSII are transferred to molecular oxygen on the acceptor-side of PSI and oxygen is reduced to superoxide anion radicals (O_2^-). Hydrogen peroxide (H_2O_2), which was produced by dismutation of the superoxide anion radical, reacts with reduced iron in iron-sulfur centers to form hydroxyl radical ($^{\cdot}OH^-$) (Sonoike et al. 1997). The hydroxyl radical thus produced destroys the iron-sulfur centers (Sonoike et al. 1995). Thus, PSI photoinhibition is derived from the electron flow from PSII. The addition of DCMU, an inhibitor of PSII or DBMIB, an inhibitor of PQ oxidation, completely suppresses PSI photoinhibition in intact leaves (Sonoike et al. 1996).

A recent paper suggested that the regulated PSII photodamage-repair cycle protects PSI from photodamage (Tikkanen et al. 2013). PSII photodamage in the absence of repair reactions markedly decreased the extent of PSI photoinhibition in the FL (Fig. 3), which would result from limitation of linear electron flow and decreases in the formation of ROS. These indicate that PSII photodamage certainly protects PSI. Does protection of PSI by the regulated PSII photoinhibition occur in the situation where both photodamage and repair occur, namely in the absence of lincomycin? The $k_{rec,PSII}$ values in the FL-1200/30 and FL-1200/240 were lower than that in the CL-1200. The $k_{pi,PSII}$ in these FL also were lower than that in CL-1200. Inhibition of the PSII repair by the ROS formed by PSI in the light can explain these low $k_{rec,PSII}$ values in the FL-1200/30 and FL-1200/240 (Nishiyama and Murata 2014). Therefore, these results indicate that the PSII photodamage-

repair cycle would not be the regulator of protection of PSI. Rather the repair cycle would be controlled by the conditions for PSI photoinhibition. Protection of PSI by the PSII photoinhibition may occur depending on the amount of ROS at the level of PSI.

When the relationships between the $k_{pi,PSII}$ and excess energy calculated from chlorophyll fluorescence measurements were examined, the $k_{pi,PSII}$ in the FL-1200/30, FL-1200/240 and CL-1200 were almost proportional to the excess energy (Fig. 6). The $k_{pi,PSII}$ in the FL-240/30 and CL-240 were deviated from this relation. The deviation of $k_{pi,PSII}$ from the regression line may also reflect insufficient activation of the photoprotective mechanisms. Further studies have to be required.

Involvement of PSII reaction center quenching

In addition to the dissipation of excess excitation energy in the PSII antenna system, there is another ΔpH-dependent NPQ process within the PSII reaction center, sometimes referred to as reaction center quenching (Krause 1988; Krause and Weis 1991; Walters and Horton 1993). I have proposed that the reaction center quenching may serve as an effective response to fluctuating light, because this quenching is reversible enough to respond quickly to light fluctuations and disappear according to the activation of the Calvin-Benson cycle, and can be extensive during first several seconds of illumination to slow down electron flow. Furthermore, it is economical

compared to irreversible quenching of PSII photoinhibition. However, the FL used in this study did not induce the reaction center quenching, even when PSII photoinhibition was accelerated in the absence of lincomycin (Fig. 8). Previous reports showed that the reaction center quenching occurs at low temperatures that inhibit electron transport (Ivanov et al. 2006) or in the leaves kept in the dark (Finannzi et al. 2004). Thus, when FL is consists of longer periods of extremely-LL phases enough to inactivate the Calvin-Benson cycle and occurs under the chilling stress, the reaction center quenching may be detected.

Roles of photosynthetic alternative electron flows in regulation of the photosynthetic electron transport system in short-term FL

Abiotic and biotic stress conditions may lead to electron redirection toward alternative electron sinks. Physiological roles of the alternative electron flows have been studied, and all flows have potentials for photoprotection from environmental stresses and/or for regulation of the photosynthetic linear electron flow. In FL, alternative electron flows can be important for protection of PSI.

The PGR5 protein is proposed to be essential for protection of PSI under the fluctuating growth light (Suorsa et al. 2012). As described in Chapter 2, the PGR5-mediated CEF is essential for protection of PSI from the FLP. In this study, I used the over-expression line of PGR5 and examined the

tolerance to the FL. In agreement with the finding of Chapter 2, the over-expression line of PGR5 markedly decreased PSI photoinhibition in the FL (Fig. 11). Moreover, these results may give a hint to solve a question why PSI photoinhibition did not accelerate PSII photoinhibition in the FL. It has been suggested that PSI photoinhibition enhance PSII photoinhibition through the reduction of the electron transfer components such as PQ (Sonoike 2011). However, in this study, PSI photoinhibition by the FLP did not show the enhanced PSII photoinhibition, although the regulated PSII photodamage-repair cycle might be active. I monitored the continuous light-driven charge fluxes in photosynthetic electron transport system in response to the moderate-FL (Fig. 13). The P515 flux signal represents the rate of the effluxes via the H^+-ATPase. Although these values does not give absolute rate, the kinetics in H^+ effluxes should provide efficient information on electron flow in the FL. If PSI photoinhibition accelerates PSII photoinhibition through the reduction of the intersystem electron transport, the rates of H^+ effluxes especially in HL phases should decrease with the cycles. However, the rates did not decrease (Fig. 13). These results strongly suggest that photosynthetic alternative electron flows compensated for the H^+ effluxes and contributed to the damaged PSI complexes, avoiding the over-reduction of the intersystem. As the over-expression line of PGR5 was tolerant to FL, the PGR5-mediated CEF might contribute significantly to the compensation of electron flow through PSI, and consequently keep the photosynthetic electron transport. The largely reduced PQ pool, shown as high values of $1-qP$ in HL phases in the FL, especially in the FL-1200/30, also supports the involvement

of the CEF-PSI (Fig. 6D).

It is suggested that the NDH-mediated CEF is not essential for responses to growth FL conditions, because *crr* mutants, deficient in NDH activity completely, did not cause growth depression and *pgr5* mutant was compensated by up-regulation of the NDH-mediated CEF when these mutants were grown in the fluctuating growth light (Suorsa et al. 2012). An NDH-deficient mutant, *crr2-2*, also showed the responses almost identical to the wild type in response to the moderate-FL as has been shown in Chapter 2. Similarly, in this study, the FL-240/30 did not induce the NDH-mediated CEF since the transient increases in chlorophyll fluorescence did not occur after turning off the light (Fig. 10). However, the activities of the NDH-mediated CEF were observed in the FL-1200/30 and CL-1200. Moreover, the activity of the CEF may be higher in FL-1200/30 than that in CL-1200. These results indicate that the electron flow in the linear electron pathway at a very high rate would drive the NDH-mediated CEF under the stressful conditions, although it remain unknown that the NDH-mediated CEF plays a role in protecting PSI from photoinhibition. Differences in the modes of light fluctuation exert distinct effects on regulation of the photosynthetic electron transport system.

When thylakoids become leaky, the flash-induced P515 signal should show the fast decay kinetics similar to Fig. 12B even in the dark-adapted leaf. Therefore, my results indicate that the H^+-ATPase was not inactivated during the FL treatments.

Concluding remarks and future scopes

I anew confirmed that fluctuating light is a potent stress factor, and that the target of the FLP is mainly PSI. The differences in the modes of the FL did not influence the degree of the PSI photoinhibition. However, this does not mean that the responses of photosynthetic electron transport system to FL-240/30 were identical to those to FL-1200/30 or FL-1200/240. When plants are exposed to the FL-240/30, the activation of the photoprotective processes, such as excess energy dissipation in the PSII antenna system and the photosynthetic control at the Cyt b_6/f complex, are insufficient to respond rapidly to the sudden increases in PPFD. This "insufficiency" of these processes in FL-240/30 may lead to PSI photoinhibition to a degree similar to those in FL-1200/30 and FL-1200/240. In contrast, FL-1200/30 and FL-1200/240 induced high activities of photoprotective processes and alternative electron flows such as CEF-PSI. It is most important to note that differences in modes of light fluctuation have distinct effects on regulation of the photosynthetic electron transport system, even if the degree of photoinhibition is the same. In this study, I indicate that moderate FL would be more stressful than the FL involving high PPFD. It is also important to stress that PSI photoinhibition did not accelerate PSII photoinhibition in the FL used in this study. Photosynthetic alternative electron flows, especially the PGR5-mediated CEF, may compensate the electron flow through PSI to

keep safely the whole electron transport.

The light environment in the field, are variable because so many modes of light fluctuation are present. As I have indicated in this study, different fluctuating light regimes provide different effects on regulation of the photosynthetic electron transport system. To understand accurately the responses to the fluctuating light in the field, I have to evaluate photosynthesis under various fluctuating light conditions. In addition, plants grown in the field are always exposed to drastic light fluctuations in PPFD. If they do not acclimate to such fluctuating light conditions, not only PSII photoinhibition but also PSI photoinhibition of the plants in the field should be more serious. I need to examine whether the photosynthetic electron transport system acclimates to the drastically fluctuating growth light. I also need to identify which of the factors, NPQ, the photosynthetic control, the PGR5-mediated CEF, or others, is responsible for the effective response to the fluctuation of light in various time scales. Finally, I have to evaluate using the field-grown plants.

3.5. Table

Table 1 Summary of the fluctuating- and continuous-light used in this study.

Light treatment	abbreviation	PPFD (HL/LL)	Cycle (min/min)
Moderate fluctuating light	FL-240/30	240/30	2/2
Continuous moderate light	CL-240	240	
High fluctuating light	FL-1200/30	1200/30	2/2
Continuous high light	CL-1200	1200	
Highest fluctuating light	FL-1200/240	1200/240	2/2

Table 2 Rate constants of photodamage of PSII ($k_{pi,PSII}$) determined in the presence of lincomycin, and those of recovery of PSII from photodamage ($k_{rec,PSII}$) in the fluctuating light and continuous light.

	PSII				
	Continuous-ML	**Moderate-FL**	**Continuous-HL**	**High-FL**	**Highest-FL**
$k_{pi,PSII}$ (min^{-1})	0.00139 ± 0.00002	0.00107 ± 0.00007	0.00558 ± 0.00009	0.00399 ± 0.00008	0.00417 ± 0.00003
$k_{rec,PSII}$ (mim^{-1})	0.0763 ± 0.0007	0.0832 ± 0.0005	0.0618 ± 0.0006	0.0483 ± 0.0007	0.0367 ± 0.0002

$k_{pi,PSII}$ was obtained from the time-courses of F_v/F_m during illumination in the presence of lincomycin (Fig. 3). Similarly, k_{rec} was estimated from time-courses of F_v/F_m in leaves during illumination in the absence of lincomycin (Fig. 2).

3.6. Figures

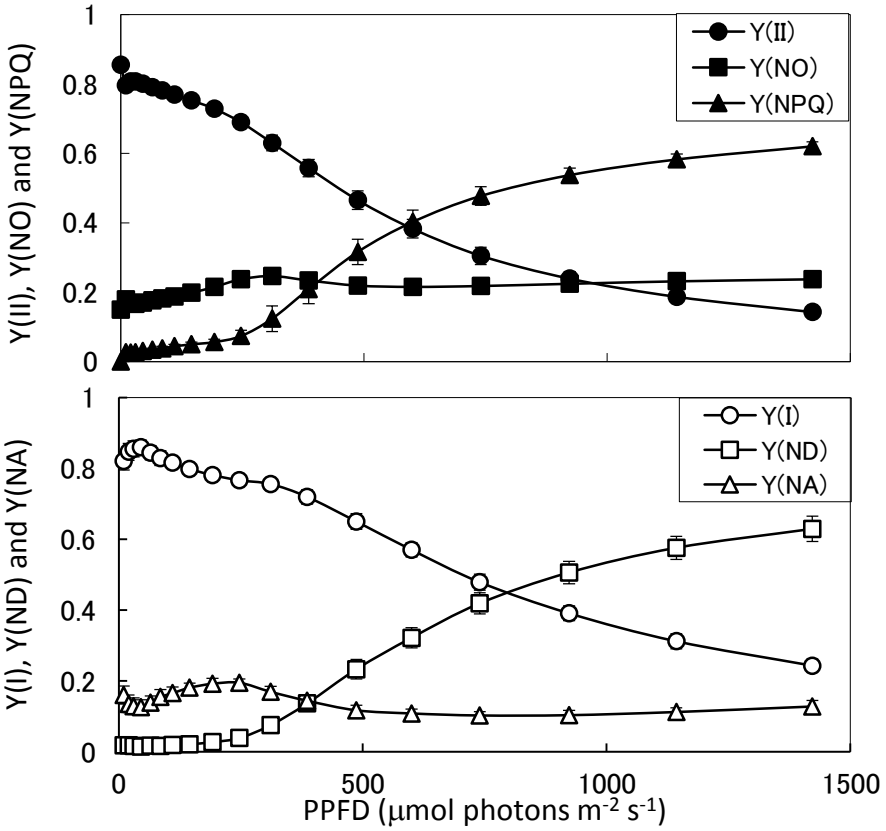


Figure 1. Responses of photosynthetic quantum yields of PSII and PSI in leaves of col-0 to continuous light at various PPFDs. The fluorescence parameters, the effective PSII quantum yield, Y(II) (circle), the quantum yield of non-regulated energy dissipation, Y(NO) (square), and that of regulated energy dissipation, Y(NPQ) (triangle), are shown. For energy captured by PSI pigments, the quantum yield of the PSI photochemistry, Y(I) (circle), that of the non-photochemical energy dissipation due to the donor-side limitation of PSI, Y(ND) (square), and that of non-photochemical energy dissipation due to the acceptor-side limitation of PSI, Y(NA) (triangle), are shown. Only PSII parameters include the values in the dark (PPFD = 0 μmol photons m^{-2} s^{-1}). Measurements were made in room air. The values represent the mean ± SD (n = 4).

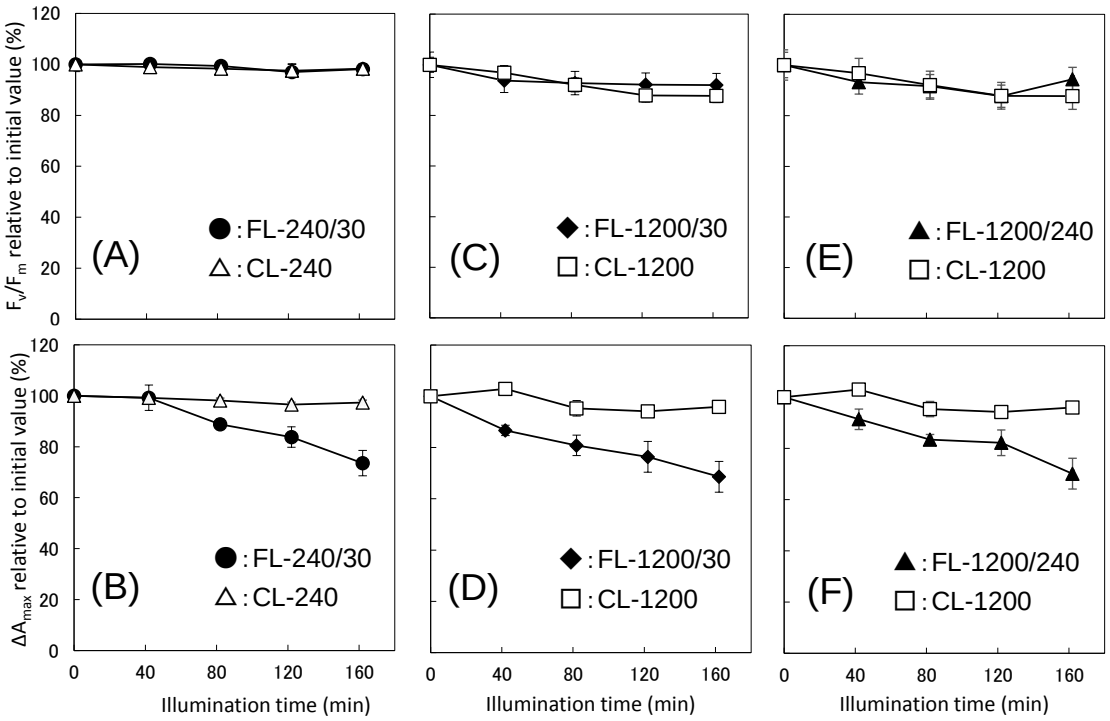


Figure 2. Effects of the fluctuating light (closed symbol) and continuous light (open symbol) on photoinhibition of PSII (A, C and E) and PSI (B, D and F) in the leaves of the col-0 plants in the absence of lincomycin. Three fluctuating light regimes included the FL-240/30 (circle), FL-1200/30 (diamond) and FL-1200/240 (triangle). Two continuous light were CL-240 (triangle) and CL-1200 (square). Following the light treatments for 42, 82, 122 and 162 min, the fractions of the functional PSII and PSI reaction center, F_v/F_m and ΔA_{max}, respectively, were determined after dark adaptation for 30 min. Data were normalized to the initial values measured in the dark before light treatments. The data of the CL-1200 in (C) and (E), and in (D) and (F) are the same, respectively. Measurements were made in room air. The values represent the mean ±SD (n = 4 - 6).

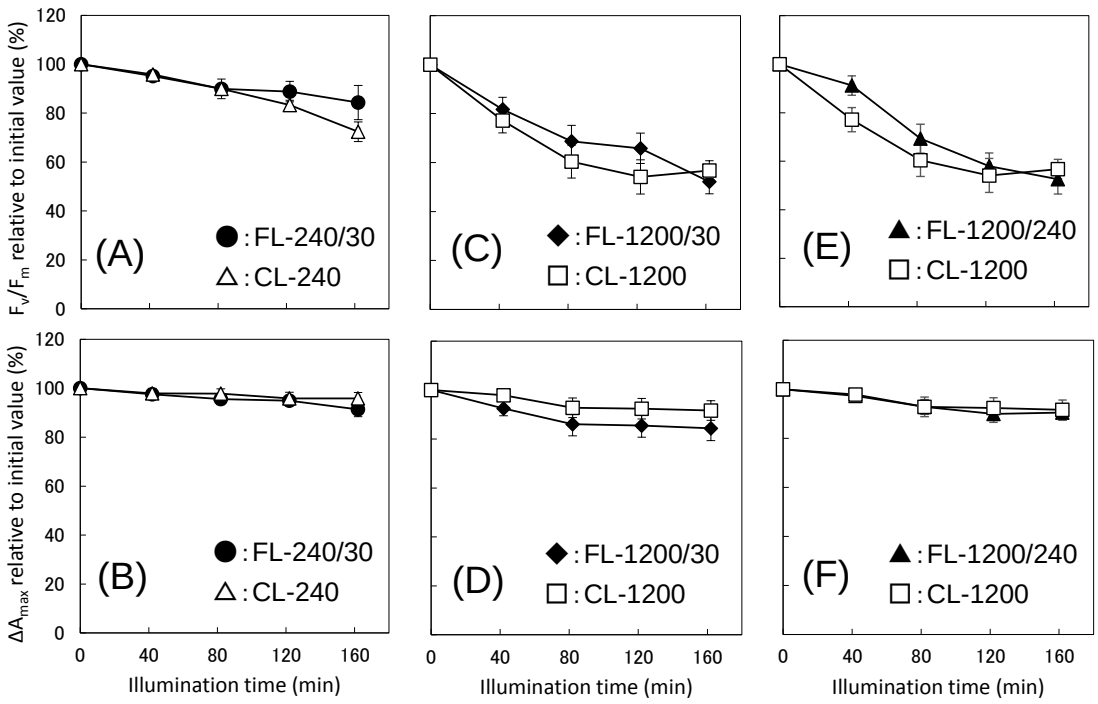


Figure 3. Effects of the fluctuating light (closed symbol) and continuous light (open symbol) on photodamages to PSII (A, C and E) and PSI (B, D and F) in the leaves of the col-0 plants in the presence of lincomycin. Light treatments and measurement protocols were same as in Fig. 2. FL-240/30 (circle); FL-1200/30 (diamond); FL-1200/240 (triangle); CL-240 (triangle); CL-1200 (square). Data were normalized to initial values measured in the dark before light treatments. The data of the CL-1200 in (C) and (E), and in (D) and (F) are the same, respectively. Measurements were made in room air. The values represent the mean ±SD (n = 3 - 6).

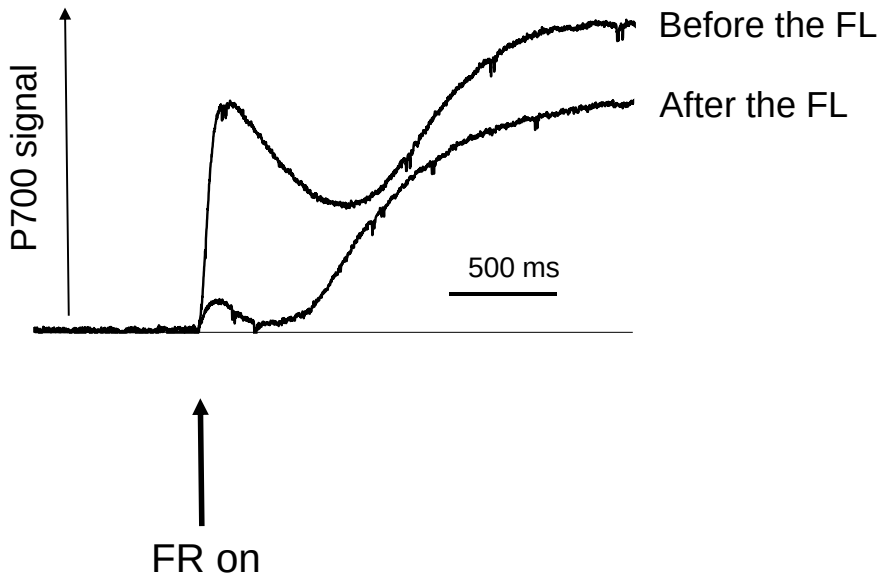


Figure 4. Effects of PSI photoinhibition on the far-red light induced $P700^+$ signal. Steady-state values of P700 signals under the illumination of far-red light were compared before and after the moderate-FL treatment for 162 min. The leaf was dark-adapted for 30 min before far-red illumination. The represented data are shown. Six set of measurements were carried out for six leaves, independently.

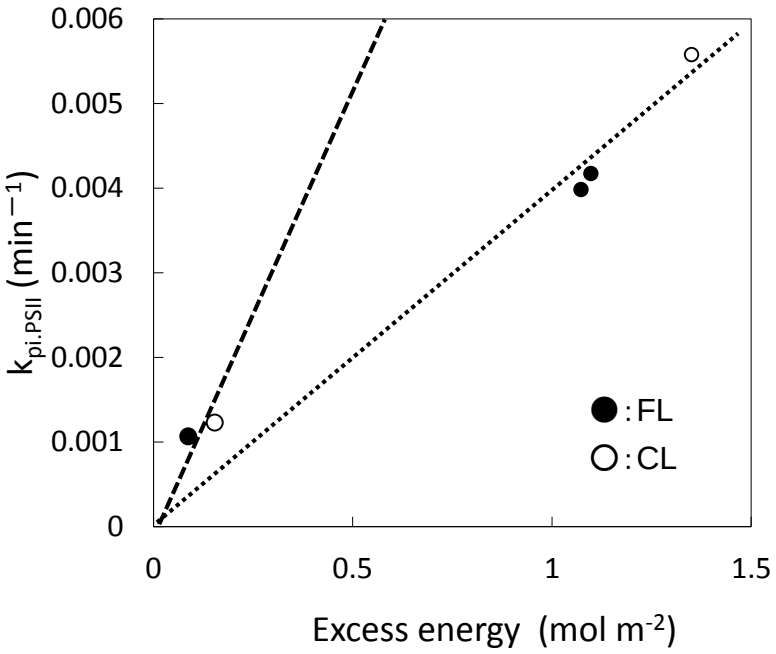


Figure 5. Relationship between $k_{pi,PSII}$ and total amount of the excess energy with different light treatments of the fluctuating- (FL, ●) and continuous-light (CL, ○).

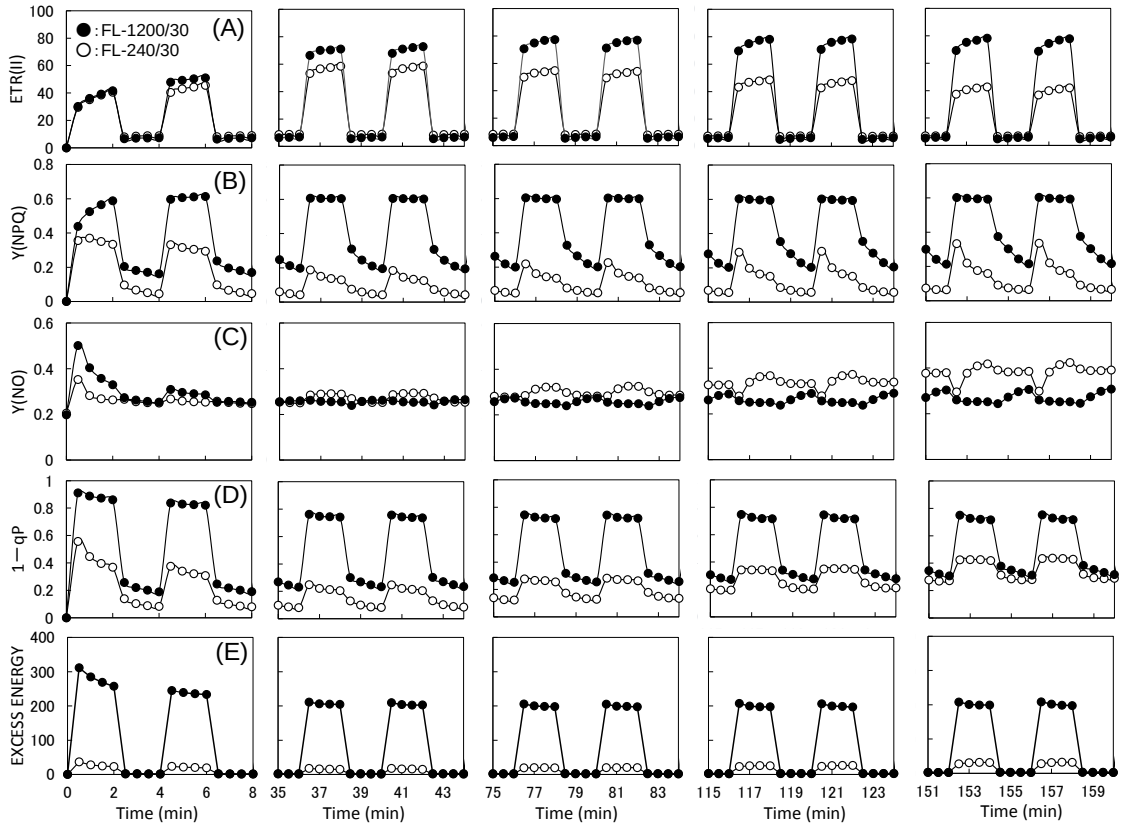


Figure 6. Changes in the PSII parameters in the FL-1200/30 (closed symbol) and FL-240/30 (open symbol) for 162 min. The light treatments were applied to the leaves after the dark treatment for 30 min. PSII parameters; ETR(II) (A), Y(NPQ) (B), Y(NO) (C), 1−qP (D), Excess energy (E). Data for 8 min were depicted from five parts during the light treatment for 162 min. Measurements were made in ventilated room air. In this series of experiments, lincomycin was not present. The values represent the mean (n = 4 - 6).

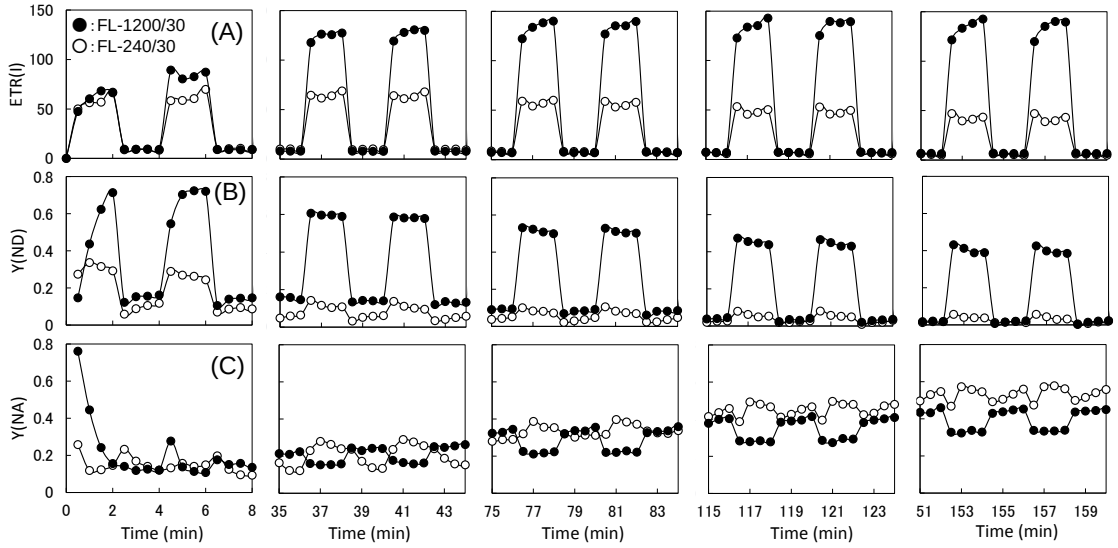


Figure 7. Changes in PSI parameters in the high-FL (closed symbol) and moderate-FL (open symbol) for 162 min. The data was measured in parallel with the PSII parameters in Fig. 6. PSI parameters; ETR(I) (A), Y(ND) (B), Y(NA) (C). Data for 8 min were depicted from five parts during the light treatment for 162 min. Measurement were made in ventilated room air. In this series of experiments, lincomycin was not present. The values represent the mean (n = 4 - 6).

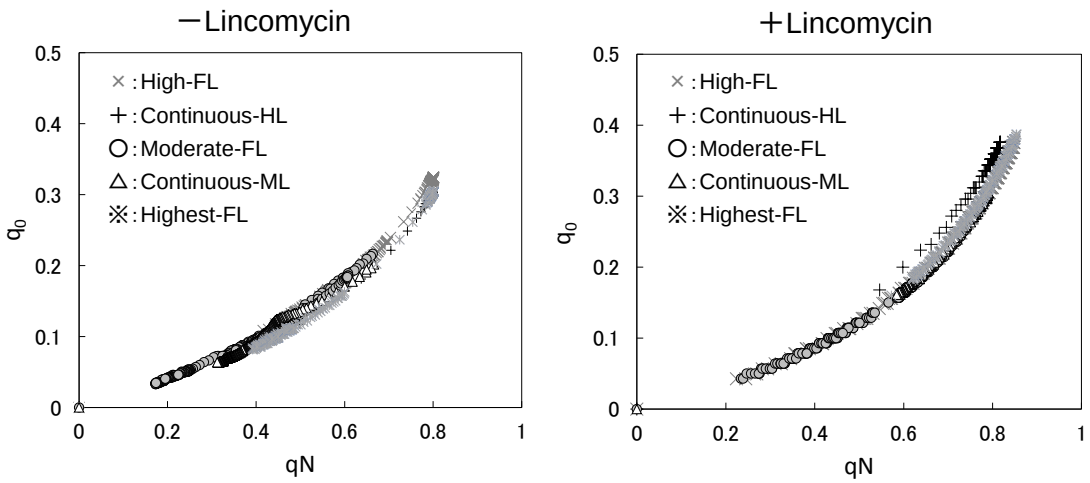


Figure 8. Relationships between q_0 and qN in various light treatments in the absence (left) and presence (right) of lincomycin. Each light treatment was applied for 162 min to the leaves after the dark treatment for 30 min. The light treatments, ×; FL-1200/30, ＋; CL-1200, ○; FL-240/30, △; CL-240; FL-1200/240, ※. The values represent the mean (n = 3 - 6).

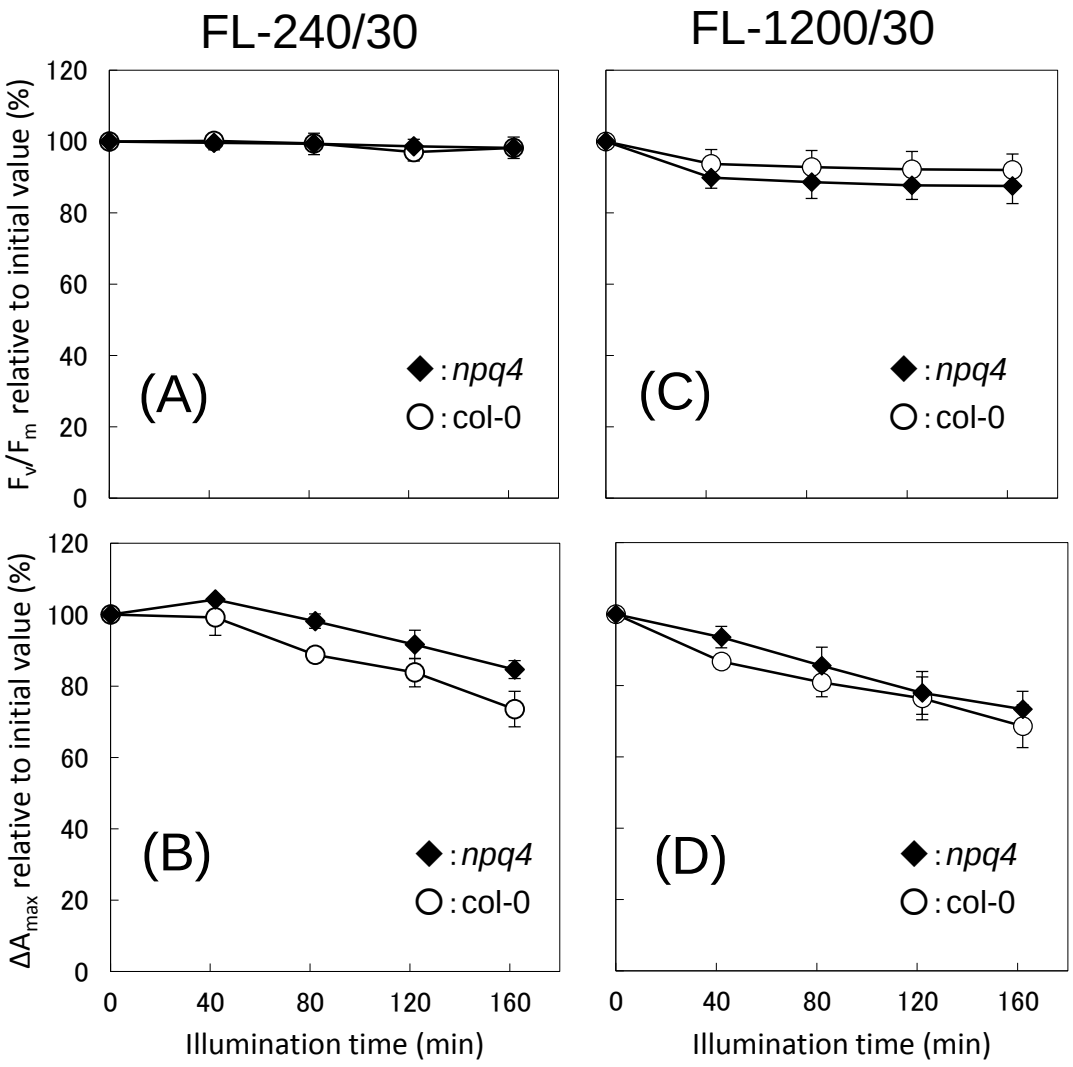


Figure 9. Effects of the FL-240/30 (A and B) and FL-1200/30 (C and D) on photoinhibition of PSII (A and C) and PSI (B and D) in the leaves of the *npq4* plants grown at the continuous light of 135 μmol photons m^{-2} s^{-1} in the absence of lincomycin. Light treatments and measurement protocols were the same for the data in Fig. 2. For comparison, the same data for col-0 (open circle) as shown in Fig. 2 are plotted. Data were normalized to the initial values measured in the dark before the light treatments. Measurements were made in room air. The values represent the mean ±SD (n = 3).

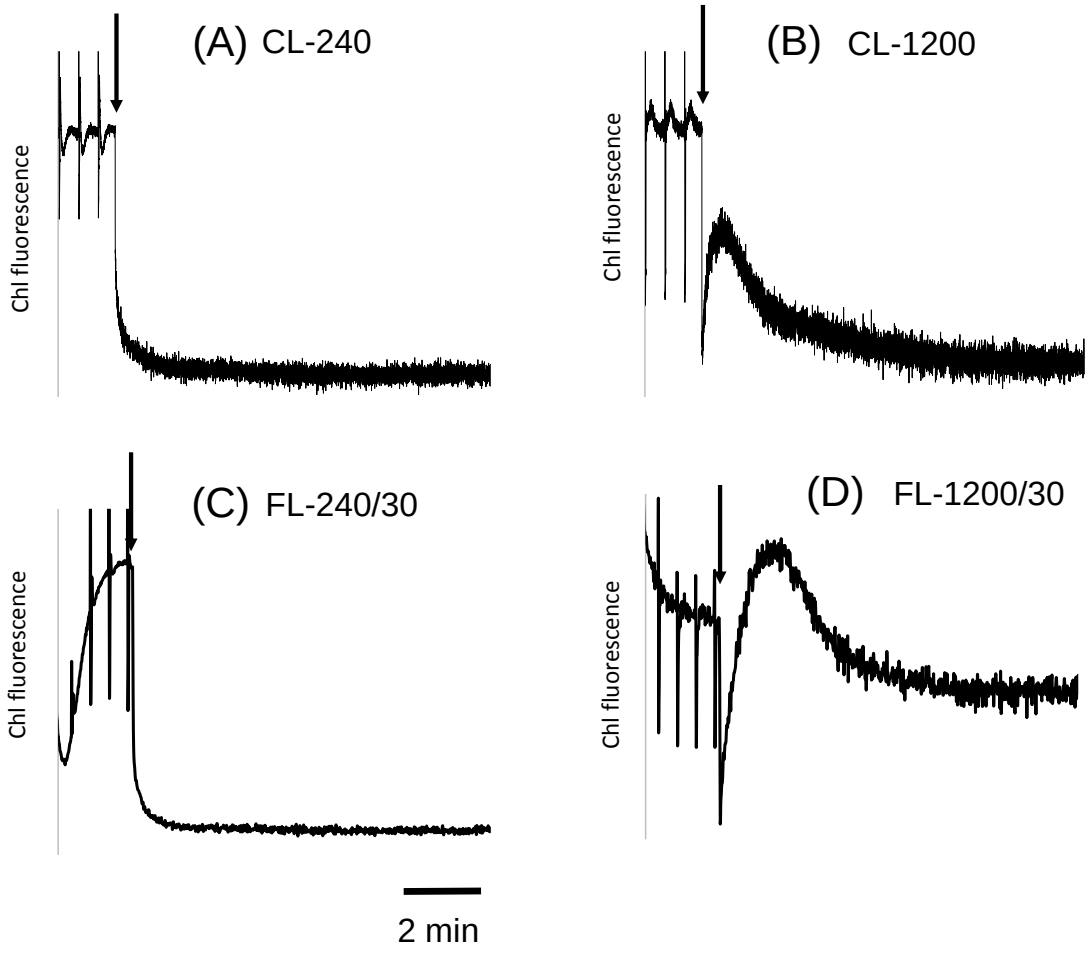


Figure 10. Analyses of the transient increase in chlorophyll fluorescence after the light treatments for 162 min; (A) CL-240, (B) CL-1200, (C) FL-240/30, and (D) FL-1200/30. Changes in chlorophyll fluorescence were monitored with the DUAL-PAM-100 (Walz). Arrows indicate the time when actinic light was off. Measurements were made in room air. The data show a typical trace (n = 3).

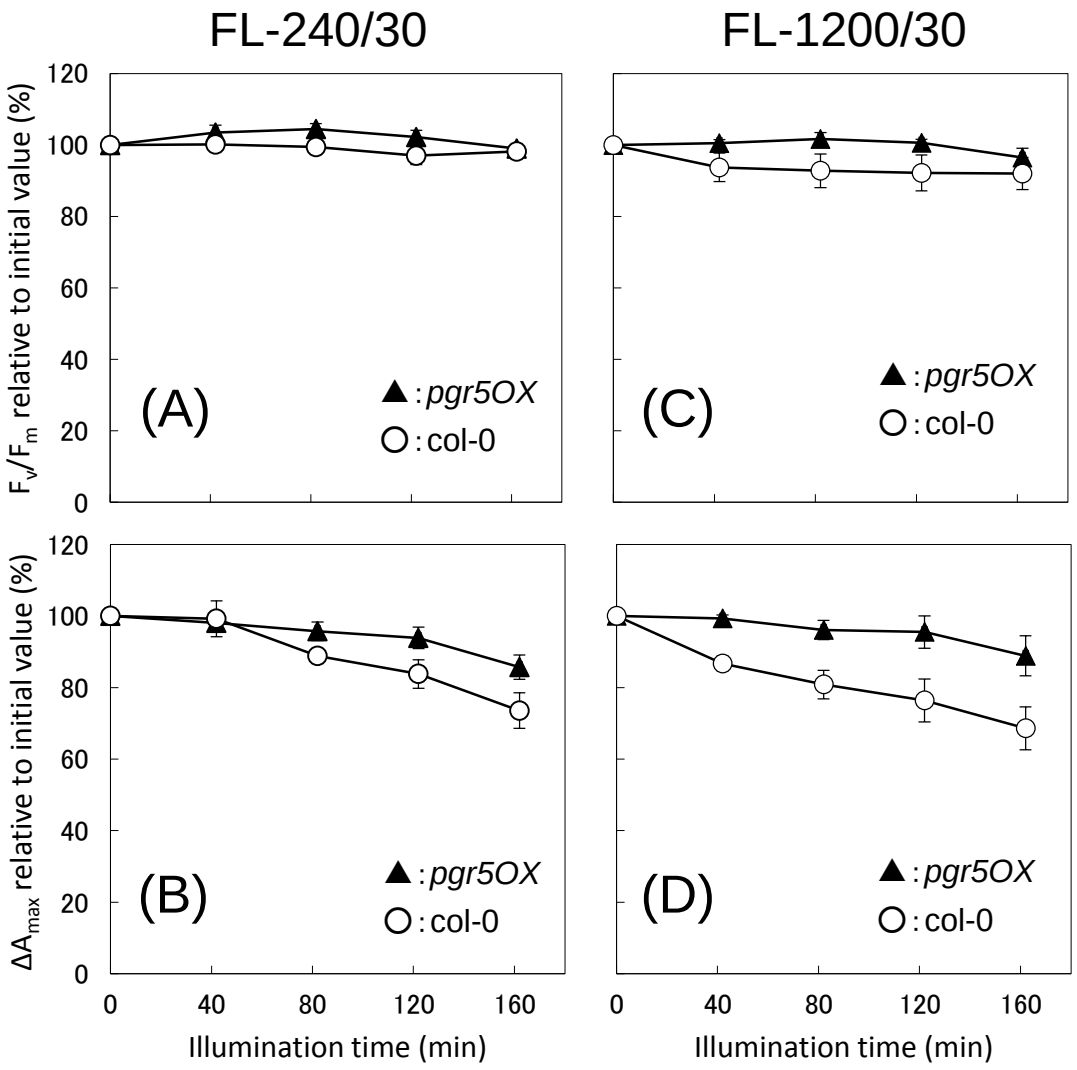


Figure 11. Effects of the FL-240/30 (A and B) and FL-1200/30 (C and D) on photoinhibition of PSII (A and C) and PSI (B and D) in the leaves of the over-expression line of PGR5 grown at the continuous light of 135 μmol photons m^{-2} s^{-1} in the absence of lincomycin. Light treatments and measurement protocols were the same as for the data in Fig. 2. For comparison, the same data for col-0 (open circle) as shown in Fig. 2 are plotted. Data were normalized to the initial values measured in the dark before light treatments. Measurements were made in room air. The values represent the mean ±SD (n = 3).

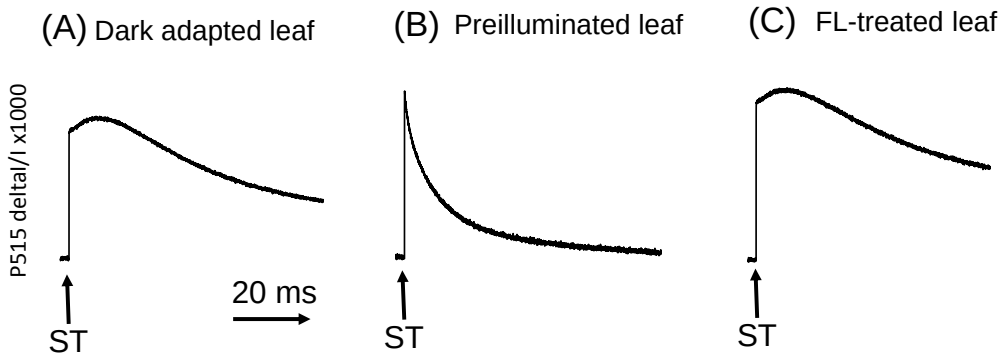


Figure 12. Typical P515 changes induced by a saturating single turnover flash (ST). (A) The leaf kept in the dark for 30 min; (B) Preilluminated leaf for 162 min in the CL-240 followed by 5 min dark. (C) Preilluminated leaf in the FL-240 for 162min followed by 30 min dark. Flash duration was chosen for 50 us. Three set of measurements were carried out for three leaves, independently.

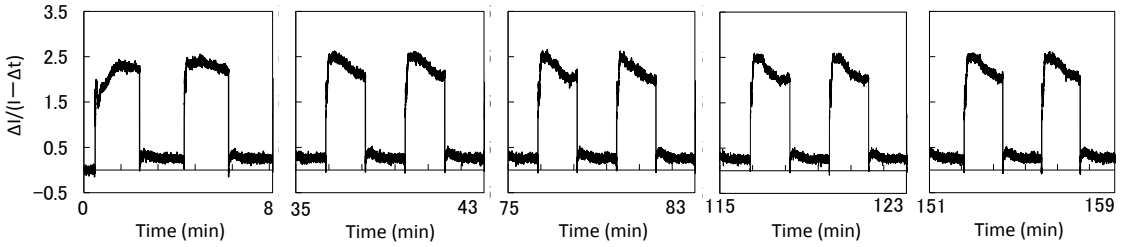


Figure 13. Continuous recording of P515 charge flux signals during the FL-240/30 for 162 min of the col-0 leaf. Actinic light was modulated as 1:1 light: dark with 20 ms on/off periods. Time integrated PPFD was set at 240 μmol photons m^{-2} s^{-1} in HL phases and 30 μmol photons m^{-2} s^{-1} in LL phases.

CHAPTER 4.

General discussion

Since the excellent studies by Pearcy and co-workers, many reports have been published concerning the effect of fluctuating light on photosynthesis. At the early stage, diversity of light conditions in canopy understories was examined intensively and significance of sunflecks for understory plants was highlighted. The study of Pearcy and co-workers revealed the great significance of sunfleck utilization in understory plants, which is associated with efficient post-illumination CO_2 assimilation (Pearcy 1990; Kirschbaum et al. 1998). Sunflecks can significantly improve carbon gain and plant growth in forest understories and carbon gain in the lower leaves in tree canopies. The study of Jansson and co-workers provided a direct ecological advantage of *A. thaliana* plants that are protected against photoinhibition in fluctuating light (Külheim et al. 2002) and opened the gate for ecological examinations of the roles of qE-quenching. The study of Aro and co-workers showed that fluctuating growth light induces photodamage particularly to PSI and that the PGR5 protein is essential for photoprotection of PSI from the acclimation experiments performed under fluctuating light (Suorsa et al. 2012). The study of Matsubara and co-workers showed that fluctuating light with different combination of duration, frequency, and intensity, elicit various acclimation responses in the plants (Alter et al. 2012).

Many botanists have not been able to relate molecular mechanisms and physiological performances. The photosynthetic apparatus is a complex system with many regulatory feed-back, feed-forward and other mechanisms operating on different timescales. Researchers should investigate not only various molecular mechanisms but also their significance in the plant physiological performance. For example, dynamics of gas exchange are often examined over a timescale of hours, providing an important set of information such as CO_2 assimilation rate and stomatal conductance. But its dynamics are integration of dynamics of many other processes including the photosynthetic electron flows in the thylakoid membrane, light capture and excitation migration in the antenna and even the interactions between chloroplasts and other organelles. Relationships between the CO_2 assimilation and other processes including the photosynthetic electron flows are to be understood on different time scales to clarify how short-term and long-term responses in fluctuating light are determined (Harbinson et al. 1990). For example, when plants are exposed to the fluctuating light with the cycle of seconds, the photosynthetic electron flow processes may not be reflected directly to CO_2 assimilation rate. When exposed to the fluctuation with the cycle of minutes, electron transport is more critical for CO_2 assimilation efficiency. In contrast, when exposed to the fluctuation with the cycle of ten minutes to hours, the response of stomatal conductance may be the most important factor.

In my studies for the doctoral thesis, I indicated that the light fluctuation itself is a potent stress factor, even when the PPFD in the HL

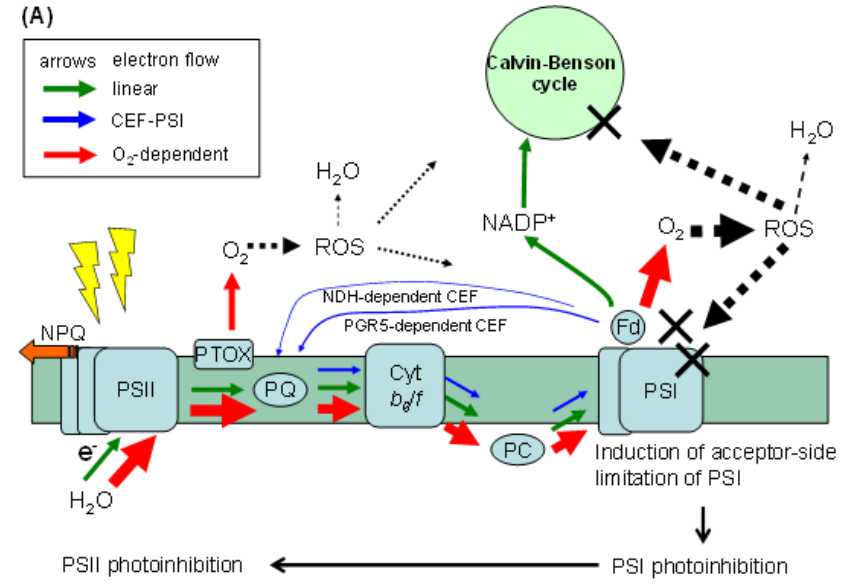
phase is moderate, and that the target is PSI (Fig. 1A). Fluctuating light causing photoinhibition is not a "single" light fluctuation such as used in most of the past studies, but are many "continuous" light fluctuations. Although previous report has shown that PSI of *A. thaliana pgr5* mutant is photoinhibited in the fluctuating growth light, I showed clearly that PSI of wild type is also markedly photoinhibited after the short-term exposure to the FL, followed by more photoinhibited with the time. Interestingly, even when PPFD in HL phase is moderate. Thus, with more drastic light fluctuations such as the FL-1200/30 and FL-1200/240, I expected more severe damage would occur. But the degrees of PSI photoinhibition by the FL1200/30 and FL-1200/240 were similar to that in the FL-240/30 for 160 min. In response to HL phases in theFL-240/30, the activation of the photoprotective processes are insufficient to respond rapidly to the sudden increases in PPFD. This "insufficiency" of the activation of these processes in HL phases in the FL-240/30 may lead to similar degree of PSI photoinhibition to that in the high-FL. In contrast, FL-1200/30 and FL-1200/240 induced high activities of photoprotective processes such as the non-photochemical energy dissipation in PSII antenna system, the down-regulation of the electron flow at the Cyt b_6/f complex and the photosynthetic alternative electron flows, especially the PGR5-mediated CEF. It is most important to note that differences in modes of light fluctuation have distinct effects on regulation of the photosynthetic electron transport system, even if the degree of photoinhibition is the same. In my study, I indicated that the moderate fluctuating light such as the FL-240/30 would be more stressful than the FL involving high PPFD (Fig.1B). It

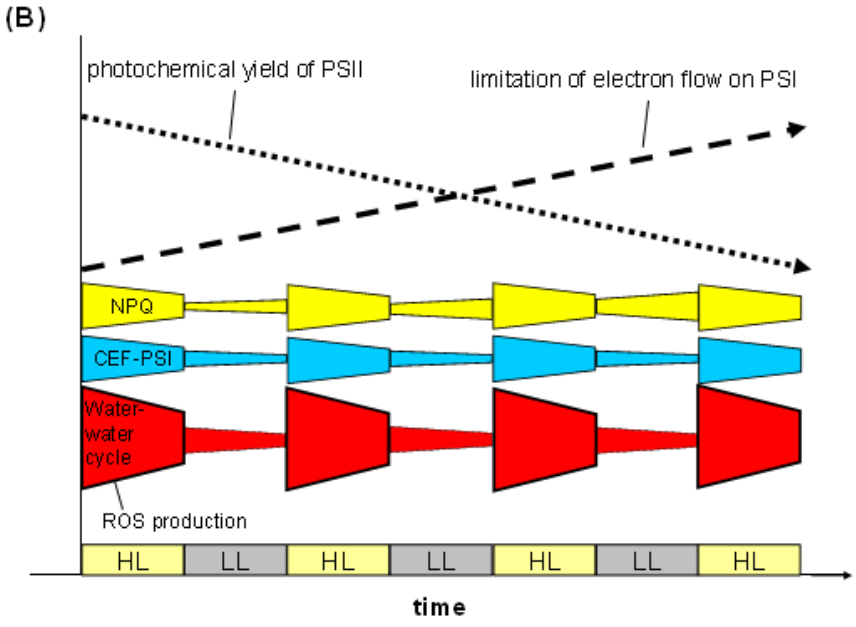
is also important to stress that PSI photoinhibition did not accelerate PSII photoinhibition in the FL used in my study. Photosynthetic alternative electron flows, especially the PGR5-mediated CEF, may compensate the electron flow through PSI to keep the whole electron transport safely.

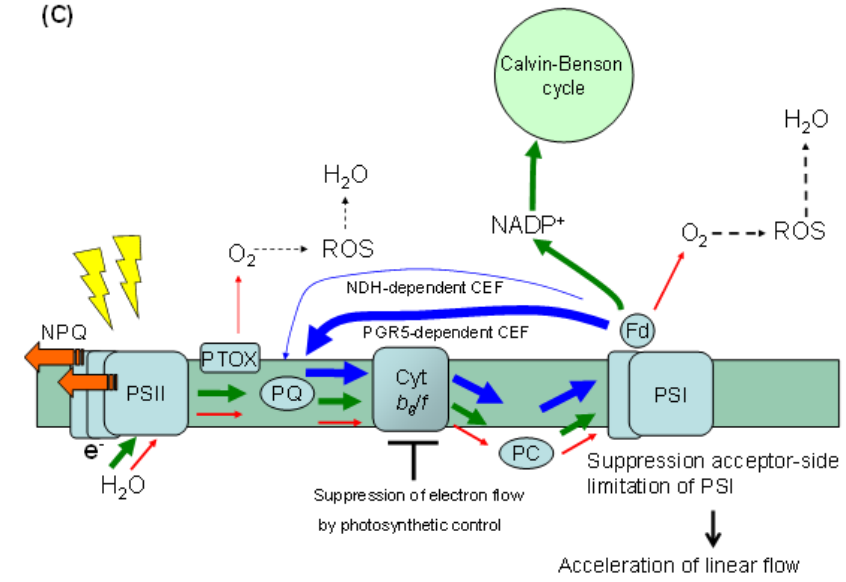
Effective responses to fluctuating light may be protection from photodamage that is caused by the rapid change in PPFD rather than the increase in the photosynthetic efficiency. For these reasons, the ability of the appropriate regulation of the photosynthetic alternative electron flows may play more important roles under the conditions with rapid light fluctuation than the dissipation of excess energy in the PSII antenna system (e.g. qE-quenching) (Fig. 1C and 1D). Furthermore, the light fluctuation not only alters the PSI/PSII ratio but also may influence energy allocation between PSII and PSI in the thylakoid membranes during acclimation because of the PSI inhibition.

Plants grown in forest understories are exposed to drastic fluctuations in irradiance. If they are able to acclimate to such fluctuating light conditions effectively, one of the mechanisms would be an enhancement of regulation ability of the CEF-PSI in response to light fluctuation. This would be achieved by the increase in the proportion of the PSI complex with the PGR5 protein. Plants may be able to avoid photodamage to PSI by increasing the ratio of PSI/PSII and/or the energy allocation to PSI in the thylakoid membranes (Jahns and Junge 1992; Yin and Johnson 2000; Suorsa et al. 2012) as was also observed in the *pgr5* mutant of *A. thaliana* grown in a continuous light as shown in chapter 2. Other photosynthetic alternative

electron flows may also play roles in the short-term response to fluctuating light. It is important to examine whether the photosynthetic apparatus in the wild-type plants acclimates to the drastically fluctuating growth light and actually become resistant to the fluctuating light. My studies in this line are under progress.


(A)
arrows electron flow
linear
CEF-PSI
O2-dependent
Calvin-Benson cycle
H2O
NADP+
O2
ROS
H2O
O2
ROS
NDH-dependent CEF
PGR5-dependent CEF
Fd
NPQ
PTOX
PSII
PQ
Cyt b6/f
PSI
e-
H2O
PC
Induction of acceptor-side limitation of PSI
PSII photoinhibition
PSI photoinhibition


(B)
photochemical yield of PSII
limitation of electron flow on PSI
NPQ
CEF-PSI
Water-water cycle
ROS production
HL
LL
HL
LL
HL
LL
HL
time


(C)
Calvin-Benson cycle
H_2O
H_2O
NADP+
O_2 ROS
O_2 ROS
NDH-dependent CEF
PGR5-dependent CEF
Fd
NPQ
PTOX
PSII
PQ
Cyt b_6/f
PSI
PC
e^-
H_2O
Suppression of electron flow by photosynthetic control
Suppression acceptor-side limitation of PSI
Acceleration of linear flow

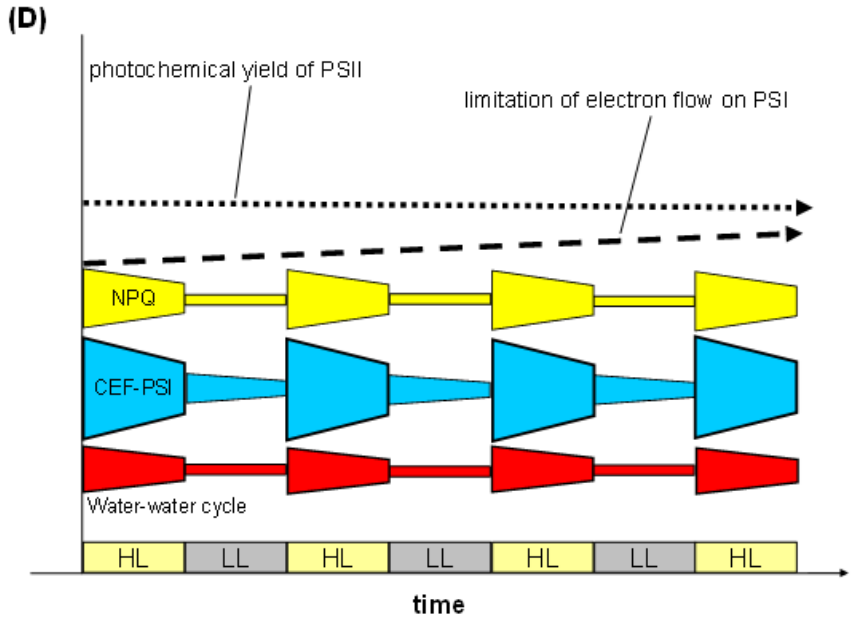

(D)
photochemical yield of PSII
limitation of electron flow on PSI
NPQ
CEF-PSI
Water-water cycle
HL
LL
HL
LL
HL
LL
HL
time

Fig. 1. Schemes of the short-term regulation of electron flows upon rapid increase in PPFD in plants.

(A) Photodamages caused by rapid increase in PPFD in the leaf with a limited activity of photoprotective processes. PSI is more sensitive to the FLP, because the acceptor-side of PSI cannot deal with a gush of electron flows caused by the rapid increase in PPFD. This results in an excess electron flow to O_2 and production of ROS. ROS thus produced eventually causes photodamage to PSI probably through the Fenton reaction.

(B) Changes in the electron transport system in the leaf with the limited activity of photoprotective processes in HL phases of the FL.

(C) Effective photoprotection by the alternative electron flows in the leaf with a high CER-PSI activity. It is important for electrons to flow to the pathways other than the water-water cycle. Increases in the activities of the alternative electron flows, especially the PGR5-mediated CEF, would prevent the acceptor-side limitation of the electron flow through PSI and photodamage to PSI, causing acceleration of the linear electron flow.

(D) Changes in the electron transport system in the leaf with a high CEF-PSI activity in HL phases of the FL.

(B and D) Solid arrows show the electron flows. Arrows in green, linear flow; arrows in blue, CEF-PSI; and arrows in red, WWC. HL and LL in the bottom bars denote high light and low light periods, respectively. The period of each HL or LL ranging from a few to several min is assumed.

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