Characterization of *Arabidopsis* mutants defective in light response

光環境応答に異常がみられるシロイヌナズナ変異体の解析

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Master thesis

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Abstract

Land plants lacks ability to move and have evolved sophisticated mechanisms allowing them to make acclimatory response to environmental changes. Light regulation of LHCII expression is one of the processes to control the light absorption in order to avoid excess light energy absorption. Despite of the accumulation of the information on the mechanism of the regulation, the physiological significance is still remained ambiguous. I characterize *FtsH6* mutant (which has been identified as a LHCII degradation mutants; Zelisko et al., 2005) and showed that the ability to dissipate excess light energy as heat (NPQ) was impaired in HL acclimated mutants, resulting in the photoinhibition of the mutant. The results indicate that LHCII degradation is significant to remove the damaged LHCII in order to protect PSII from high light stress. In addition to this mutant, the characterization of the 32-11-13 mutant, which has also a defect in NPQ, were also performed. I found that the mutant showed defect in hypocotyl elongation only under the condition where NPQ was induced at high level. I hypothesize that the mutant has a defect in blue-light signaling pathway and the defect causes both hypocotyl elongation, and high level of heat dissipation (NPQ).

Introduction

In photosynthesis, sunlight is converted into chemical energy that is stored mainly as carbohydrates and supplies basically all life on Earth with energy. In contrast to animal development, which is predominantly genetically determined, plant development is to a significant extent governed by environmental factors. Lacking the ability to move, plants have to be able to respond and acclimate to broad spectrum of growth climates and stress factors that vary at timescales from seasons to seconds.

From the beginning of the photosynthetic research, the main focus has been the functional and structural aspects behind the energy-transducing process. The progress has been significant, and the photosynthetic protein complexes can now be described at a very refined molecular level (Ferreira et al., 2004; Merchant et al., 2005; Szabo et al., 2005). Conversely, relatively little is known about the acclimating, regulatory, and protective processes that maintain high photosynthetic efficiency during ever-fluctuating and even stressful environmental conditions.

Irradiance stress occurs whenever the light energy absorbed by the photosynthetic apparatus is in excess of that required for photosynthesis and results in the photoinhibition of photosynthesis. PSII appears to be the primary site of this photo-inhibitory damage, and thus plants adjust the structure and composition of their photosynthetic apparatus to protect PSII (Szabo et al., 2005; Walter, 2005). Under these circumstances, regulation of light harvesting system is necessary to balance the absorption and utilization of light energy, thereby minimizing the chance of photooxidative damage. To achieve this protective process, one of the strategies is to regulate the amount of light-harvesting chlorophyll protein complexes. In

higher plants, the most abundant light harvesting protein, LHCII (light-harvesting chlorophyll *a*/*b* binding protein complex of photosystem II), is known to have this function. For example, a reduction in photon flux densities leads to a burst of the expression of *Lhcb* genes (encoding Lhcb proteins, the components of the LHCII), whereas high light condition decreases the level of this transcript (Tanaka et al, 2005; Walter and Horton, 1994). As a result, the LHCII proteins are degraded in order to avoid excess absorption of light energy under high-light condition, and re-synthesized under low-light condition (Bailey et al. 2001; Tanaka et al, 2005).

Despite the wealth of physiological information, the underlying molecular mechanisms for the change in the amount and composition of LHCII are still not understood. Furthermore, physiological importance of the LHCII degradation is also obscure. Genetic methods are powerful tools in physiology research because comparison of mutant and wild-type plants can define the function of the missing gene/protein. Recently, Zelisko et al. (2005) have been identified a novel Arabidopsis mutant defective in LHCII degradation during high-light acclimation. They observed that the degradation of LHCII was impaired in the *ftsH6*, in which a gene for putative FtsH protease is not functional. Although a gene responsible for the LHCII degradation was identified, the physiological information of the phenomenon was not clarified. In order to investigate the physiological significance of LHCII degradation, I determined photosynthetic performance of this mutant *in vivo*. The results show that the high-light acclimated mutants have the low ability to dissipate the excess light energy as heat (non-photochemical quenching; NPQ) protect PSII, resulting the photoinhibition of photosystem II. The physiological meanings of these results are discussed in this master thesis.

Since chlorophyll fluorescence reflects the redox state of

photosynthetic electron transport chain, it has been used as a method to acquire the information of photosynthesis activity (Schreiber et al, 1994). Recently, Niyogi et al. developed the screening system to make it possible to obtain the fluorescence imaging of seedlings with a CCD camera (Niyogi et al., 1998). Several mutants which had high chlorophyll fluorescence after high light illumination have been isolated and characterize as the mutants impaired in NPQ, which helped us to gain molecular information of the mechanism of NPQ (Niyogi et al., 1998; Niyogi, 1999; Munekage et al., 2001, 2002). In order to obtain mutants that impaired in the regulation of photosynthetic electron transport, screening experiments were performed with the chlorophyll fluorescence in our laboratory (Higuchi, Doctor Thesis, 2004). By this experiment, the 32-11-13 mutant was isolated as the mutant had a difference in chlorophyll fluorescence kinetics from the wild type plant. Since the results of chlorophyll fluorescence in *ftsH6* showed us that an effect of a mutation in light-harvesting components could be monitored by analyzing the change in parameters of chlorophyll fluorescence as a sensitive indicator, I also conducted the characterization of the 32-11-13 mutant by chlorophyll fluorescence measurements. I found that the mutant showed defect in hypocotyl elongation only under the condition where NPQ was induced at high level. The result suggests the unexpected relation between photomorphogenesis and light harvesting.

Materials and methods

Plant materials and Growth condition

Seeds of Wild type and T-DNA knockout mutant lacking FtsH6 (SALK012429; At5g15250) of *Arabidopsis thaliana* (var. Colombia; Col) were obtained from the Arabidopsis Biological Resource Center (The Ohio State University). Seeds of *Arabidopsis thaliana* (var Wassilewsija; Ws) and T-DNA populations were also obtained from the same resource center. The 32-11-13 mutant was isolated from this T-DNA population as the mutant that shows different chlorophyll fluorescence kinetics from wild type under high light condition (Higuchi, Doctor thesis, 2004). These plants were grown in Murashige and Skoog agar plates at 23°C under continuous illumination. To monitor the process of light-acclimation, seedlings of wild type (Col) and *FtsH6* mutant were grown for 20 days under low light condition (50 μmol photons m^{-2} s^{-1}; LL), and then transferred to high light condition (300 μmol photons m^{-2} s^{-1}; HL) for 4 days. As control experiments, other seedlings were grown under LL continuously without the transfer to HL condition. To compare the effect of growth light intensity, the wild type (Ws) and the 32-11-13 mutant were grown under three different photon flux densities (Dark, 50, and 150 μmol photons m^{-2} s^{-1}). Homozygous mutations of *FtsH6* mutants were confirmed by using PCR with the forward primer 5' – ATT GAT GCT GTT GGG AGA ATG – 3' and the reverse primer 5' – CCG TAG AAC CAC ATC GTT TTG –3'. Amplification of the insertion was performed by using the primer 5'- GCG TGG ACC GCT TGC AAC T –3'.

Chlorophyll fluorescence measurement

Chlorophyll fluorescence was measured by a pulse-amplitude modulation (PAM) fluorometer (Waltz, Effeltrich, Germany) with emitter detector unit ED101 as described in Schreiber et al. (Schreiber et al., 1986). Rosettes of each plant were dark adapted for 30 min before measurement. The minimum chlorophyll fluorescence (Fo) was determined by applying the measuring light (650 nm) at 0.02 μmol photons m^{-2} s^{-1}. A saturating pulse of white light at 3000 μmol photons m^{-2} s^{-1} set for 0.8 s from a light source (KL 1500; Schott, Wiesbaden, Germany) was applied to determine the maximum chlorophyll fluorescence in the dark (Fm) and during actinic light illumination (Fm'). In order to investigate the dependence of chlorophyll fluorescence on actinic light intensity, plants were measured four times with different photon flux densities of the actinic light (50, 150, 300, and 500 μmol photons m^{-2} s^{-1}). The steady state of chlorophyll fluorescence level (F) was recorded during actinic illumination. The maximum quantum yield of PSII (Fv/Fm) was calculated as (Fm – Fo) / Fm, and the quantum yield of open PSII under actinic illumination (Fv'/Fm') was calculated as (Fm'-Fo') / Fm'. Non-photochemical quenching (NPQ) was calculated as (Fm – Fm') / Fm'. Photochemical quenching (qP), which reflects the oxidized state of plastoquinone, was calculated by an equation (Fm' – F)/ (Fm' – Fo'). 1-qP indicates the reduced state of the plastoquinone. The effective quantum yield of PSII (ϕII) was calculated as (Fm' – F) / Fm'.

Northern blot analysis

Total leaf RNA was isolated using Rneasy® Plant Mini Kit (QIAGEN). Isolated RNA was subjected to electrophoresis on 0.9%

agarose gels containing 3.0% formaldehyde (2 μg of total RNA was loaded per lane), and blotted onto positively charged nylon membranes (Pall Corporation Biodyne PLUS), and hybridized with digoxigenin (DIG)-labeled DNA probe. To make a DIG-labeled DNA probe, the Lhcb1 was amplified by PCR and then labeled with DIG by DIG DNA Labeling Kit (Roche). The primers used for the PCR amplification were 5'- GCC TCA ACA ATG GCT CTC TCC T - 3' and 5'- AAC AAA GTT GGT GGC GAA GG - 3'. Aliquot of the DIG-labeled probe hybridization solution was used at 50°C and then Lhcb1 transcripts were detected by DIG Nucleic Acid Detection Kit (Roche) as chemiluminescence.

PAGE and Western blot analysis

To isolate intact chloroplasts, leaves were chopped in a homogenizer (Polytron, Kinematica, Lucerne, Switzerland) with ice-cold buffer (50 mM HEPES-KOH, pH 7.5, containing 0.33 M D-Sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA and 5 mM Sodium ascorbate). The mixture was then filtrated through the 20 μm nylon mesh and the filtrate was centrifuged, and the pellet was washed and resuspended in the same buffer. Chloroplast proteins corresponding to 5 μg proteins were separated on 12% SDS-polyacrylamide gels and stained with Coomassie Brilliant Blue R250. For western blot analysis, chloroplast proteins corresponding to 1 μg proteins were transferred to a polyvinylidene difluoride membrane using a semidry blotting system. Immunoblot detection was performed using an enhanced chemiluminescence system (ECL; Amersham Biosciences). Lhcb1 antibody was obtained from Agrisera (Sweden).

Chlorophyll quantification

To quantify chlorophyll, pigments of leaves were extracted into N, N-dimethylformamide (DMF) for 3 h at 4°C in dark. Amounts of Chl *a* and Chl *b* were determined spectrophotometrically using the equations of Porra et al. (1989).

Results

Characterization of the mutant defective in LHCII degradation process

Degradation of Lhcb1 during high light acclimation did not occurred in the mutant

Only one mutant, which has the T-DNA insertion in the second exon of *FtsH6* (At5g15250) gene, was reported to have a defect in LHCII degradation in the past (Figure 1, Zelisko et al., 2005, Sakamoto et al., 2003). First I confirm that the *FtsH6* mutants are not able to degrade LHCII during high light (HL) acclimation at our experimental condition. The amount of Lhcb1 protein was compared between HL-acclimated wild types (WT) and the *FtsH6* mutants by western blot analysis (Figure 3). As shown in Figure 3, the amount of Lhcb1 protein in WT decreased significantly after the transfer to HL condition for 4 days (compare WT HL0 and HL4), whereas that of the mutants remained at the initial LL level (compare Mutant HL0 and HL4). LHCII degradation should also affect the ratio of chlorophyll (Chl) *a* to chlorophyll *b*, since chlorophyll *b* binds specifically to the light-harvesting proteins while chlorophyll *a* binds to both light-harvesting proteins and photosystem core proteins. For this reason, Chl *a*/*b* ratio was widely used as an indicator of the change in the amount of LHCII (Bailey et al., 2001; Tanaka et al., 2005; Walter and Horton, 1994). In WT, Chl *a* and Chl *b* decreased about 50% and 56% of the initial LL value after HL-acclimation, respectively (Table 1). This results in 12% increase in Chl *a*/*b* ratio. In the mutants, however, Chl *a* and Chl *b* decreased about 31% and 30% respectively, resulting in no significant change in Chl *a*/*b* ratio. These results suggest that the degradation of LHCII is impaired in the

FtsH6 mutants. On the contrary to these differences between WT and the mutant after the shift to HL condition, the growth of the HL-acclimated plants (Figure 2, upper panel) did not show any differences from that of LL-grown plants (Figure 2, lower panel).

Light regulation of lhcb1 mRNA level was normal in the mutant

In order to investigate whether the mutation of the *FtsH6* mutant affects the *lhcb*1 mRNA level, northern blot analysis was performed (Figure 4). As in the past reports (Tanaka et al., 2005; Walter and Horton, 1994), the *lhcb*1 mRNA level of WT decreased significantly after transferred to HL condition and gradually increased during HL acclimation, while the mRNA level of LL acclimated plants (control) did not change. Similarly, the mRNA level of the *lhcb*1 decreased after transferred to the HL condition, which indicates that the deletion of the *FtsH6* mutant has no effect on light regulation of *lhcb*1 transcription.

Photosynthesis activity and Non-photochemical quenching were lower in the mutant

Light energy absorbed by chlorophyll molecule in a leaf can undergo one of three fates: 1) it can be used to drive photosynthesis (photochemistry), 2) excess energy can be dissipate as heat or 3) it can be re-emitted as light, i.e. chlorophyll fluorescence (Figure 0). These three processes occur in competition, such that any increase in the efficiency of one will result in a decrease in the yield of others. Hence, by measuring the yield of chlorophyll fluorescence, information about changes in the efficiency of photochemistry and heat dissipation can be obtained. In order to gain this information, measurement of chlorophyll fluorescence was performed with WT and mutant plants. There are three parameters of

chlorophyll fluorescence (Fv/Fm, ϕII, and NPQ) that show significant differences between WT and mutants.

A change in Fv/Fm reflects the maximum quantum yield of PSII and used as a sensitive indicator of plant photosynthetic performance. Therefore, lower values of this parameter will be observed when the plant has been exposed to stress, indicating in particular the phenomenon of photoinhibition. As shown in Figure 5A, Fv/Fm was significantly lower in HL acclimated mutants indicating the susceptibility of the mutant to photoinhibition by HL stress.

ϕII is the most useful parameter to measure the efficiency of photosystem II (PSII) photochemistry under specific light condition. This parameter indicates the effective quantum yield of electron transport determined at PSII. Therefore, it can give a measure of the rate of linear electron transport at steady state level. As shown in Figure 5B, HL-acclimated mutants showed significant lower ϕII at lower photon flux densities (PFD) of actinic light (50 and 150 μmol photons $m^{-2}s^{-1}$), while there is no significant difference at higher PFD. These results show that the linear electron flow was inhibited only at lower PFD of actinic light in the HL acclimated mutants.

Another widely used parameter is qP, which gives a measure of the proportion of PSII reaction centers that are oxidized (so called open PSII centers). An alternative expression of this parameter is 1-qP, the proportion of the centers that are reduced (so called closed PSII centers). ϕII and qP can be interrelated by a third parameter, Fv'/Fm', which measures the intrinsic efficiency of open PSII centers. According to these relations, ϕII can be calculated as qP x Fv'/Fm', which describe the change in the efficiency of electron transport (ϕII) by the proportion of open reaction

centers (qP) and their potential quantum efficiency (Fv'/Fm'). As shown in Figure 6 A, Fv'/Fm' of the HL-acclimated mutants were lower from other plants at lower PFD of actinic light, resulting in the lower value of ϕII. At higher PFD, however, Fv'/Fm' of the HL-acclimated mutants did not show the significant difference from that of WT. qP was not so much affected in both the LL-grown and the HL-acclimated mutants (Figure 6 B).

The decrease of Fv'/Fm' could be induced either by the decrease of maximum quantum yield (Fv/Fm) or by the increase of energy dissipation as heat. NPQ, which is the measure of energy dissipation, of the HL-acclimated mutants showed significant lower value especially under the actinic light at higher photon flux densities when compared with that of WT (Figure 6 C).

Characterization of 32-11-13 mutants

Defect of hypocotyl elongation in the 32-11-13 mutants

As described above, chlorophyll fluorescence is one of the effective indicators to investigate the performance of photosynthesis. For this reason, in our laboratory, screening of 1600 Arabidopsis T-DNA mutants with chlorophyll fluorescence has been performed (Higuchi, Doctor thesis, 2004). The 32-11-13 mutant was one the mutants isolated by this screening method that shows different chlorophyll fluorescence kinetics from WT. I first compared the growth of this mutant plants under several different light conditions (dark, 50, and 150 μmol photons $m^{-2}s^{-1}$). There was no significant difference between WT and the mutants grown under dark and 150 μmol photons m^{-2} s^{-1} light conditions, whereas the mutants grown under 50 μmol photons m^{-2} s^{-1} (Low Light; LL) show 2-fold elongation of hypocotyl length (Figure 7 and Table 2). Overall morphology

and the shape of leaves were not so much different between WT and the mutant both under 50 μmol photons m^{-2} s^{-1} and 150 μmol photons m^{-2} s^{-1} (Figure 8). The contents of Chl *a* and *b* slightly decreased only in LL grown mutants compared to wild type plants, but it did not accompany the change in Chl *a*/*b* ratio (Table 2).

Non-photochemical quenching was higher in the 32-11-13 mutants

In order to investigate the performance of photosynthesis in detail, PAM measurement was performed. Fv/Fm did not show any difference between WT and the mutant (Figure 9 A). On the other hand, mutants grown under 50 μmol photons m^{-2} s^{-1} showed lower Fv'/Fm' and higher NPQ compared with WT especially when determined with actinic light at high PFD (Figure 10 A). On the cotrary to Fv'/Fm', the value of qP was low with actinic light at high PFD (Figure 11 A). Since ϕII calculated as qP x Fv'/Fm', low Fv'/Fm' and high qP results in slight difference of ϕII between WT and the mutant (Figure 9 B). There is no difference in any parameters between WT and mutants grown under 150 μmol photons m^{-2} s^{-1} (Figure 9 A, C, Figure 10 B, and Figure 11 B).

Discussion

Physiological significance of LHCII degradation

By the characterization of the *FtsH6* mutants, a new insight into LHCII degradation has been provided. Although the main function of LHCII is energy capture and transfer, it is also involved in the light regulatory process. In this respect, LHCII is known to be regulated in a light dependent manner both at transcriptional and post-transcriptional level (Bailey et al., 2001; Flachmann et al., 1995; Tanaka et al., 2005; Walter and Horton, 1994). However the underlying molecular mechanisms are still rather ambiguous. Since LHCII is highly conserved in higher plants (Jansson, 1994 1999), it has been assumed to have physiological significance in the regulation of LHCII for plant survival especially under changing light condition. Despite of this widely accepted expectation, no evidence has been gained so far to answer this question. Recently, Zelisko et al. has been identified the mutant defective in LHCII degradation (Zelisko et al. 2005). They initially characterized a proteases possibly involved in the LHCII degradation and showed that the protease has the properties similar to FtsH proteases. They subsequently analyzed several *FtsH* mutants (*ftsH5*, *ftsH6*, and *ftsH11*) and eventually identified FtsH6 as the one that is responsible in LHCII degradation. Despite of this identification of the protease, the significance of the degradation process of LHCII is still remained to be obscure. Thus, I conducted the physiological characterization of this mutant.

The most unexpected result in this study is that the mutant showed the defect in the quantum yield of photosynthetic electron transport only under low actinic light condition (Figure 5 B). The yield of electron

transfer is normal under higher actinic light condition. At first glance, this seems quite strange since the mutant is defective in the degradation of LHCII under high light condition, not under low light condition (Figure 3). This discrepancy was solved when I examined the photosynthetic characteristics more in detail.

Usually, excess light absorption induces the NPQ mechanism for heat dissipation in order to protect photosynthetic apparatus from photo-oxidative damages (Demmig-Adams et al., 1996; Muller et al. 2001; Niyogi 1999), resulting in the increase of the NPQ, a chlorophyll fluorescence parameter. However, the induction of NPQ by high light was severely suppressed in the mutant (Figure 6 C). Usually, the decrease of energy dissipation leads to the increase of quantum yield of photosynthesis because of the competition between the two processes. In the case of this mutant, the maximum quantum yield is low after the shift to high light judging from the low Fv/Fm value (Figure 5 A). As a result, effective quantum yield of PSII (Fv'/Fm', Figure 6 A) and of electron transport (ϕII, Figure 5 B) of the mutant is not so much different from that of Wt under high actinic condition, since the decrease of energy dissipation (NPQ) is compensated by the decrease of maximum quantum yield of PSII (Fv/Fm). When the actinic light is low, however, NPQ is not much induced both in WT and in the mutant so that the decrease of Fv/Fm directly causes the decrease of Fv'/Fm' (Figure 6 A). Thus, the photosynthetic electron transport is lower in the mutant than in WT only under low actinic light condition (Figure 5 B).

Then, next question arises. The mutant showed two phenotypes under high light condition: the decrease in Fv/Fm and the decrease of NPQ. Which is the cause and which is the result? Since the non-photochemical quenching is one of the protective mechanisms against photoinhibition

(Muller et al., 2001), it is reasonable to assume that the decrease of NPQ is the first event, and that subsequently leads to the photoinhibition of PSII resulting in the decrease of Fv/Fm. It should be noted that NPQ is induced only under high light, and that Fv/Fm of the mutant decreased only under high light condition (Figure 5 A).

In Arabidopsis, nine *FtsH* homologues are targeted to the chloroplast. Mutations in *FtsH2* (*VAR2*) and *FtsH5* (*VAR1*) give rise to a yellow variegated phenotype, whereas other *FtsH* mutants including the *ftsH6* show no phenotypic difference from WT (Nixon et al., 2004). The maximum yield of photochemistry in PSII measured by chlorophyll fluorescence indicated that *var1* and *var2* are highly sensitive to photoinhibitory light exposure, suggesting that VAR1 and VAR2 play a critical role in the photoprotection of chloroplast. Further analysis *in vivo* suggested that one of the substrates for VAR2 is a photo-damaged D1 protein of the PSII reaction center (Bailey et al., 2002). Degradation of the D1 protein is key process in the repair cycle of photo-damaged PSII (Aro et al., 1993; Melis et al., 1999), and FtsH proteases appears to function in this process with DegP proteases (Itzhaki et al., 1998; Haussuhl et al., 2001). Based on these past reports, the results in this study are able to interpret the defect of LHCII degradation as the defect of LHCII repair process driven by FtsH6. I suppose that severe photoinhibition of PSII is induced in the mutants because of the existence of NPQ-defective LHCII, which is to be removed by the process of LHCII degradation in normal plants. This means that LHCII degradation is required to remove the photo-damaged LHCII in order to avoid further damage from secondary photoinhibition. This new insight will contribute to describe the physiological importance of LHCII, since it indicates the presence and significance of the LHCII turnover for the plant survival in stressful environment.

Relationship between hypocotyl elongation and alteration in photosynthesis

The 32-11-13 mutant was originally isolated as the one that showed altered induction kinetics of chlorophyll fluorescence (Higuchi, Doctor thesis, 2004). Unexpectedly, I found that this mutant showed long hypocotyl phenotype than the wild type only under photon flux density at 50 μmol photons m^{-2} s^{-1} (Figure 7 and Table 2). Photosynthesis performance was also significantly different from the wild type only when grown under the same light condition. In the dark, as well as under photon flux density at 150 μmol photons m^{-2} s^{-1}, I could not observe specific phenotype either in morphology or in photosynthesis. As far as I know, this is the first report of mutants defective in both photomorphogenesis and photosynthesis under specific light condition.

The Arabidopsis hypocotyl is a useful model for investigating the regulation of plant growth. Hypocotyl elongation in Arabidopsis is the result of cell expansion that is under both environmental and hormonal controls. In the absence of light, seedlings undergo skotomorphogenic development. The cotyledons remain closed, an apical hook is formed, and the hypocotyl becomes greatly elongated. Light induces the photomorphogenic developmental program resulting in cotyledon expansion, leaf development, the initiation of photosynthesis, and limited hypocotyl growth. Light is undoubtedly the most important environmental factor that inhibits hypocotyl elongation (Vandenbussche et al., 2005). Receptors responsive to different spectral ranges mediate this response. Mutations in cryptochromes (blue light receptors) and phytochromes (red and far-red light receptors) cause long hypocotyl phenotypes (Ahmad et al,

1993; Somers et al., 1991). In addition to light, all of the known plant hormones have also been implicated to function in the control of hypocotyl elongation. Brassinosteroids, auxin, and gibberellins promote hypocotyls growth, whereas cytokinins and abscisic acid (ABA) have inhibitory effects (Clouse et al., 1996; Chaudhury et al., 1993; Jacobsen et al., 1993; Romano et al., 1995). Therefore, it is able to postulate that plant photoreceptors or/and plant hormones are involved in the hypocotyl phenotype of the 32-11-13 mutants.

Among these photomorphogenic mutants, reports on the mutants of blue-light photoreceptors seem to help us to understand the phenotypes of 32-11-13 mutants. Arabidopsis has two types of genes encoding for the blue-light receptor proteins, cryptochrome (CRY1 and CRY2) and phototropin (PHOT1 and PHOT2) (Chen et al., 2004; Briggs et al., 2002). These are known to mediate blue light responses, including hypocotyl development and phototropism. The mutants defective in cryptochromes show long hypocotyl phenotype (Ahmad et al., 1998; Jackson et al., 1995 Lin et al., 1996). In contrast to *CRY1*-deficient mutants, which showed long hypocotyl phenotype at any photon flux densities, the length of the hypocotyl was greatly reduced at higher photon flux densities in *CRY2*-deficient mutants. Furthermore, the expression of CRY2 are reported to be rapidly down regulated by blue light in a photon flux density dependent manner, indicating that cryptochrome 2 functions primarily under low light during the early development of seedlings (Lin et al., 1998). Considering these reports, it is tempting to assume that the 32-11-13 mutants have a defect in blue-light mediated hypocotyl elongation process.

If this is true, what is the reason for the higher non-photochemical quenching of chlorophyll fluorescence in the mutants? NPQ is the parameter describes the rate of heat dissipation of excess light energy. This

process is known to driven by three xanthophyll molecules (violaxanthin; V, antheraxanthin; A, and zeaxanthin; Z). Under excess light condition, violaxanthin is converted rapidly via the intermediate antheraxanthin to zeaxanthin by violaxanthin de-epoxidase, leading to the dissipation of absorbed light energy as heat. On the contrary, this reaction is reversed under low light condition by the conversion of zeaxanthin to violaxanthin catalyzed by zeaxanthin epoxidase (Demmig-Adams et al., 1996). Several mutants that are defective in this xanthophyll cycle and the NPQ process are known. For example, *npq2* are known to have a defect in the Z to V conversion resulting in the accumulation of zeaxanthin and shows higher value of NPQ. On the other hand, *npq1* which has a defect in V to Z conversion lacks zeaxanthin under high light and shows NPQ-deficient phenotype (Dall'Osto et al., 2005; Niyogi, 1999). In this respect, the 32-11-13 mutant, which has high NPQ than WT (Figure 10), seems to accumulate the excess level of zeaxanthin. The absorption spectrum of zeaxanthin, closely matches the action spectrum for blue light-stimulated stomatal opening, which was known to be regulated mainly by phototropins (Kinoshita et al., 2001). It was also reported that inhibition in zeaxanthin formation by the inhibitor of violaxanthin de-epoxidase, dithiothreitol, inhibits blue-light stimulated opening of stomata (Srivastava et al., 1995). Furthermore, *npq1* also showed a defect in stomata opening (Frechilla et al., 1999; Talbott et al., 2003; Zeiger, 2000, Zeiger et al., 2002). Based on these knowledge, it has been assumed that zeaxanthin is able to act as a blue-light photoreceptor. Taking all into consideration, it is able to postulate that the defect of the 32-11-13 mutant cause the accumulation of surplus zeaxanthin, which induce higher NPQ induction, and this excess zeaxanthin affects the hypocotyl elongation as a blue-light receptor for the reaction. In order to test this possibility, the investigation of zeaxanthin accumulation in

32-11-13 mutants as well as the observation of hypocotyl phenotype of other NPQ mutants should be examined in near future.

References

Ahmad M, Cashmore RA (1993) HY4 gene of *A. thaliana* encodes protein with characteristic of a blue-light photoreceptor. Nature 366; 162-166

Ahmad M, Jarillo JA, Cashmore RA (1998) Chimeric proteins between cry1 and cry2 Arabidopsis blue light photoreceptor indicate overlapping function and varying protein stability. Plant Cell 10; 197-207

Aro EM, Virgin I, Andersson B (1993) Photoinhibition of photosystem II Inactivation, protein damage and turnover. Biochim. Biophys. Acta 1143; 113-134

Bailey S, Walters RG Jansson S (2001) Acclimation of *Arabidopsis thaliana* to the light environment; the existence of separate low light and high light responses. Planta 213; 794-801

Bailey S, Thompson E, Nixon PJ, Horton P, Mullineaux CW Robinson C, Mann NH (2002) A critical role for the Var2 FtsH homologues of *Arabidopsis thaliana* in the photosystem II repair cycle *in vivo*. J. Biol. Chem. 277; 2006-2011

Briggs WR, Christie MJ (2002) Phototropin 1 and 2: versatile plant blue-light receptors. Trends Plant Sci. 7; 204-210

Chaudhury AM, Letham S, Craig S, Dennis ES (1993) *amp1*- a mutant with high cytokinin levels and altered embryonic pattern, faster vegetative growth, constitutive photomorphogenesis and precious flowering. Plant J. 4; 907-916

Chen M, Chory J, and Frankhauser C (2004) Light signal transduction in higher plants. Annu. Rev. Genet. 38; 87-117

Clouse SD (1996) Molecular genetic studies confirm the role of

brassinosteroids in plant growth and development. Plant J. 10; 1-8

Demmig-Adams B, Adams WW (1996) The role of xanthophylls cycle carotenoids in the protection of photosynthesis. Trends Plant Sci.1; 21-26

Dall'Osto L, Caffarri S, Bassi R (2005) A mechanism of nonphotochemical energy dissipation, independent from PsbS, revealed by a conformational change in the antenna protein CP26. Plant Cell 17; 1217-1232

Frechilla S, Zhu J, Talbott LD, Zeiger E (1999) Stomata from npq1, a zeaxanthin-less Arabidopsis mutant, lack specific response to blue light. Plant Cell Phsysiol. 40; 949-954

Ferreira, KN, Iverson TM, Maghlaoui K, Barber J, Iwata S (2004) Architecture of the photosynthetic oxygen-evolving center. Science 303; 1831-1838

Flachmann R, Kuhlbrand W (1995) Accumulation of plant antenna complexes is regulated by post-transcriptiona mechanisms in Tobacco. Plant Cell 7; 149-160

Garcia-Lorenzo M, Zelisko A Jackowski G, Funk C (2005) Degradation of the main photosystem II light-harvesting complex. Photochem. Photobiol Sci. 4; 1065-1071

Govindjee (2002) A role for a light-harvesting antenna complex of phtosystem II in photoprotection. Plant Cell 14; 1663-1668

Haussuhl K, Andersson B, Adamska I (2001) A chloroplast DegP2 protease performs the primary cleavage of the photodamaged D1 protein in plant photosystem II. EMBO J. 20; 713-722

Itzhaki H Naveh L, Lindahl M, Cook M Adam Z (1998) Identification and characterization of DegP a serine protease associated with the luminal side of the thylakoid membrane. J. Biol. Chem. 273;

7094-7098

Jackson JA, Jenkins IG (1995) Extension-growth responses and expression of flavonoid biosynthesis genes in the *Arabidopsis hy4* mutant. Planta 197; 233-239

Jacobsen SE, Oiszewski NE (1993) Mutation at the *SPINDLY* locus of *Arabidopsis* alter Gibberellin signal transduction. Plant Cell 5; 887-896

Jansson Stefan (1994) The light-harvesting chlorophyll *a*/*b*-binding proteins. Biochim. Biophys. Acta 1184; 1-19

Jansson Stefan (1999) A guide to the Lhc genes and their relatives in Arabidopsis. Trends Plant Sci. 4; 236-240

Kinoshita T, Doi M, Suetsugu N, Kagawa T, Wada M, Shimazaki (2001) phot1 and phot2 mediated blue light regulation of stomatal opening. Nature 414; 656-660

Lin C, Ahmad M, Cashmore RA (1996) Arabidopsis cryptochrome is a soluble protein mediating blue-dependent regulation of plant growth and development. Plant J. 10; 893-902

Lin C, Yang H, Guo H, Mockler T, Chen J, Cashmore AR (1998) Enhancement of blue-light sensitivity of Arabidopsis seedlings by a blue light receptor cryptochrome 2. Proc. Natl. Acad. Sci. USA 95; 2686-2690

Maxwell K, Johnson GN (2000) Chlorophyll fluorescence - practical guide. J. Exp. Bot. 51; 659-668

Merchant S, Sawaya MR (2005) The light reactions: A guide to recent acquisitions for the picture gallery. Plant Cell 17; 648-663

Muller Patricia, Lie PX, Niyogi KK (2001) Non-photochemical quenching. A response to excess light energy Plant Physiol. 125; 1558-1566

Munekage Y, Hojo M, Meurer J, Endo T, Tasaka M, Shikanai T (2002) PGR5 is involved in cyclic electron flow around photosystem I and is essential for photoprotection in Arabidopsis. Cell 110; 361-371

Munekage Y, Takeda S, Endo T, Jahns P, Hashimoto T, Shikanai T (2001) Cytochrome b_6f mutation specifically affects thermal dissipation of absorbed light energy in Arabidopsis. Plant J. 28; 351-359

Nixon PJ, Barker M, Boehm M, Vries de R, Komenda J (2004) FtsH-mediated repair of the photosystem II complex in response to light stress. J. Exp. Bot. 56; 357-363

Niyogi KK (1999) Photoprotection revisited: genetic and molecular approaches. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50; 333-359

Niyogi KK, Grossman AR, Bjorkman O (1998) Arabidopsis mutants define a central role for the xanthophylls cycle in the regulation of photosynthesis energy conversion Plant Cell 10; 1121-1134

Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophyll *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim. Biophys. Acta 975; 385-394

Romano PC, Robson RHP, Smith H, Estelle M, Klee H (1995) Transgenic-mediated auxin overproduction in *Arabidopsis*: hypocotyl elongation phenotype and interactions with the *hy6-1* hypocotyl elongation and axr1 auxin-resistent mutants. Plant Mol. Biol. 27; 1071-1083

Sakamoto W, Zaltsman A, Adam h, Takahashi Y (2003) Coordinated regulation and complex formation of YELLOW VARIEGATED 1 and YELLOW VARIEGATED 2, chloroplastic FtsH metalloproteases involved in the repair cycle of photosystem II. Plant Cell 15; 2843-2855

Schreiber U (1986) Detection of rapid induction kinetics with a new type o high-frequency modulated chlorophyll fluorometer. Photosynth. Res. 9; 261-272

Schreiber U, Bilger W, Neubauer C (1994) Ecophysiology of photosynthesis. Ecological studies 100. 45-70

Somers DE, Sharrock RA, Tepperman JM, Quail PH (1991) the hy3 long hypocotyls mutant of arabidopsis is deficient on phytochrome B. Plant Cell 3; 1263-1274

Srivastava A, Zeiger E (1995) The inhibitor of zeaxanthin formation, dithiothreitol, inhibits blue-light- stimulated stomatal opening in *Vicia faba*.Planta 196; 445-449

Szabo I, Bergantino E M, Giacometti GM (2005) Light and oxygenic photosynthesis: energy dissipation as a protection mechanism against photo-oxidation. EMBO reports. 6; 629-634

Talbott LD, Shmayevich IJ, Chung Y, Hammad JW, Zeiger E (2003) Blue light and phytochrome-mediated stomatal opening in the *npq1* and *phot1 phot2* mutants of Arabidopsis. Plant Physiol. 133; 1522-1529

Tanaka R, Tanaka A (2005) Effects of chlorophyllide *a* oxygenase overexpression on light acclimation in *Arabidopsis thialiana*. Phtosynth. Res. 85; 327-340

Vandenbussche F, Verbelen JP, Straeten DVD (2005) Of light and length: regulation of hypocotyl growth in Arabidopsis. BioEssays

27; 275-284

Walter RG (2005) Towards an understanding of photosynthetic acclimation. J. Exp. Bot. 56; 435-447

Walter RG, Horton P (1994) Acclimation of *Arabidopsis thaliana* to the light environment: changes in composition of the photosynthesis apparatus Planta 195; 248-256

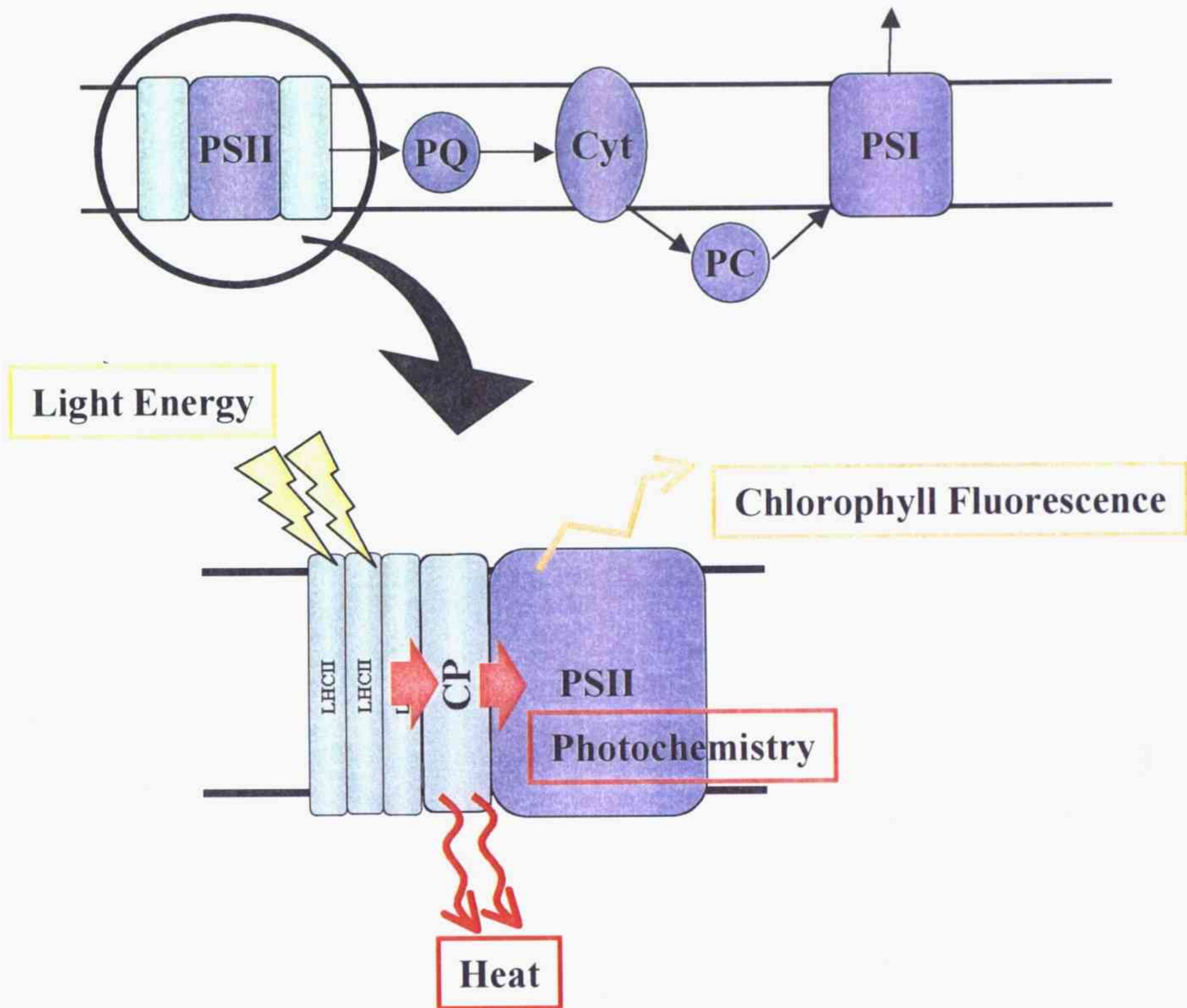
Zeiger E (2000) Sensory transduction of blue light in guard cells. Trends Plant Sci. 5; 183-185

Zeiger E, Talbott LD, Frechilla S (2002) The guard cell chloroplast: perspective for the twenty-first century. New Phyt. 153; 415-424

Zeiger E, Zhu Jianxin (1998) Role of zeaxanthin in blue light photoreception and the modulation of light-CO_2 interactions in guard cells. J. Exp. Bot. 49; 433-442

Zelisko A, Garcia-Lorenzo M, Jackwoski G, Jansson S, Funk C (2005) AtFtsH6 is involved in the degradation of the light-harvesting complex II during high-light acclimation and senescence. Proc. Natl. Acad. Sci. USA. 102; 13699-13704

Figures



Light Energy = Photochemistry + Heat + Chlorophyll Fluorescence

Figure 0. Schematic model of light energy utilization in photosystem II
Since this master thesis describes mainly about the functions of photosystem II complex, the model is focused on utilization of light energy in the complex. Abbreviations: PSII, photosystem II; PSI, photosystem I; PQ, plastoquinone; Cyt, cytochrome b_6f complex; PC, plastocyanin; LHCII, light-harvesting chlorophyll a/b binding protein complex; CP, core antenna pigment protein complex

At5g15250 (chromosome 5)

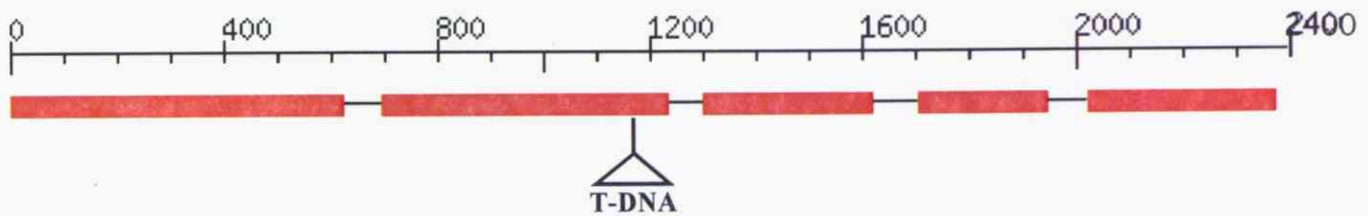


Figure 1. The T-DNA insertion site of *ftsH6*.
The T- DNA insertion site of *ftsH6* is indicated by triangle bar. The insertion was in At5g15250 *Arabidopsis thaliana* gene locus. Lines and closed boxes represent introns and exons, respectively. This figure was made according to the information of Sakamoto W. et al. (2003)

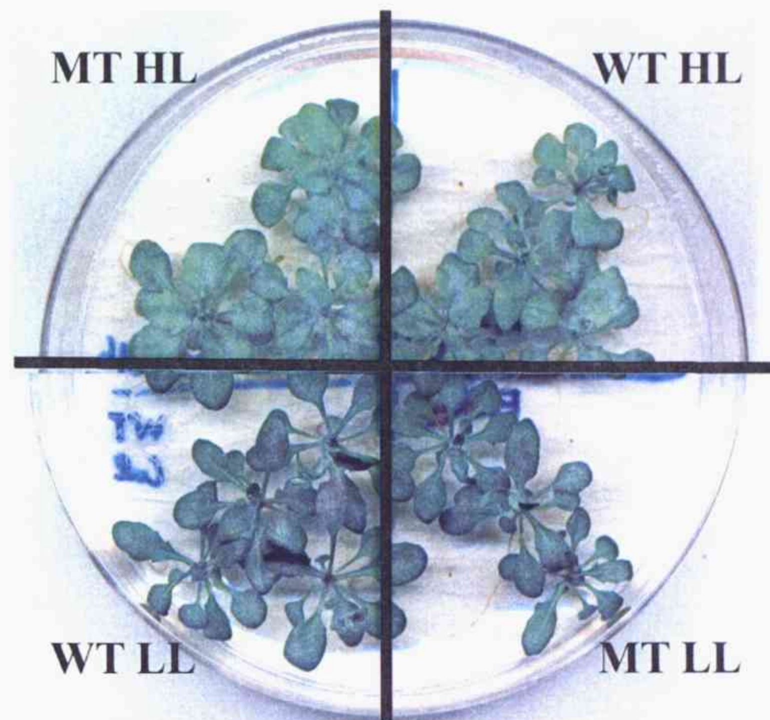


Figure 2. Growth of Wild types and mutants before and after HL-acclimation
Wild types and *FtsH6* mutants were first grown under 50 μmol photons m^{-2} s^{-1} low light (LL) condition (lower half, left; wild types, right; mutants) for 20 days, and then transferred to the 300 μmol photons m^{-2} s^{-1} high light (HL) condition (upper half, left; mutants, right; wild types) for 4days (HL-acclimation).

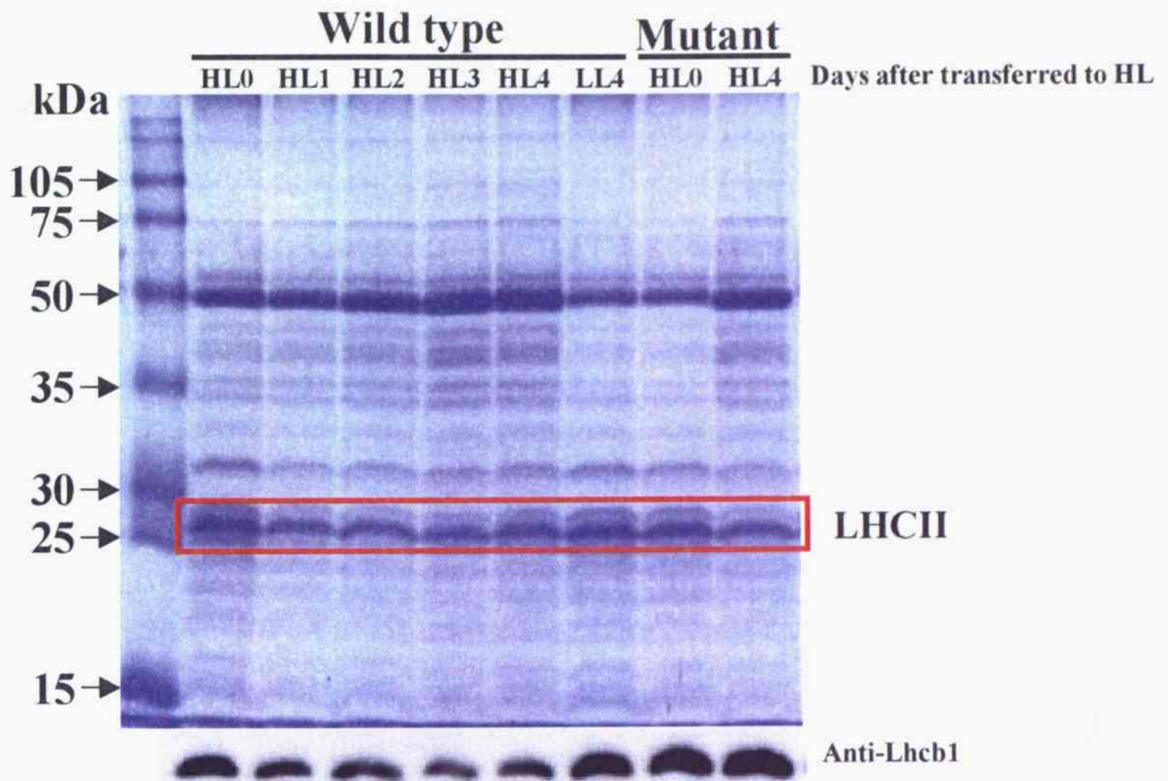


Figure 3. Western blot analysis of HL-acclimated plants

Both wild types and *FtsH6* mutants were grown as described in Figure 1, and chloroplasts were isolated every day after transferred to high light condition (HL0 ~ 4). 5 μg of total chloroplast proteins were subjected to SDS-PAGE for coomassie brilliant blue staining (upper panel), and 1 μg for western blot analysis (lower panel). Lhcb1 specific antibody was used for detection.LL-acclimated plants were use as control (LL4)

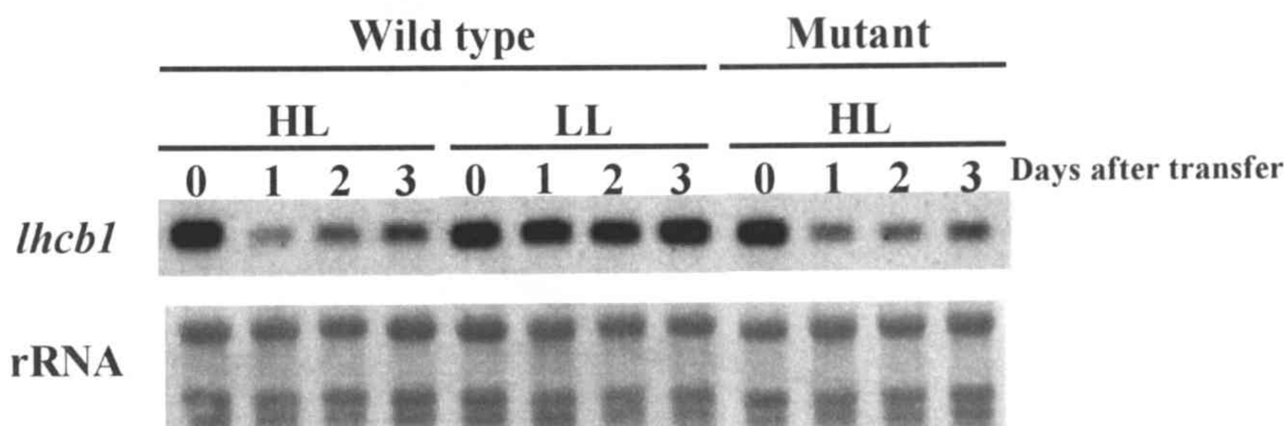


Figure 4. Northern blot analysis of HL-acclimated plants
Both wild types and *FtsH6* mutants were grown as described in Figure 1, and total leaf RNA was isolated every day after transferred to high light condition (HL Lane 0 ~ 3). LL-acclimated plants were also analyzed as control. 2 μg of total RNA was loaded in each lane and *Lhcb1* mRNA was detected. Methylene blue stained rRNA is represented as a loading control.

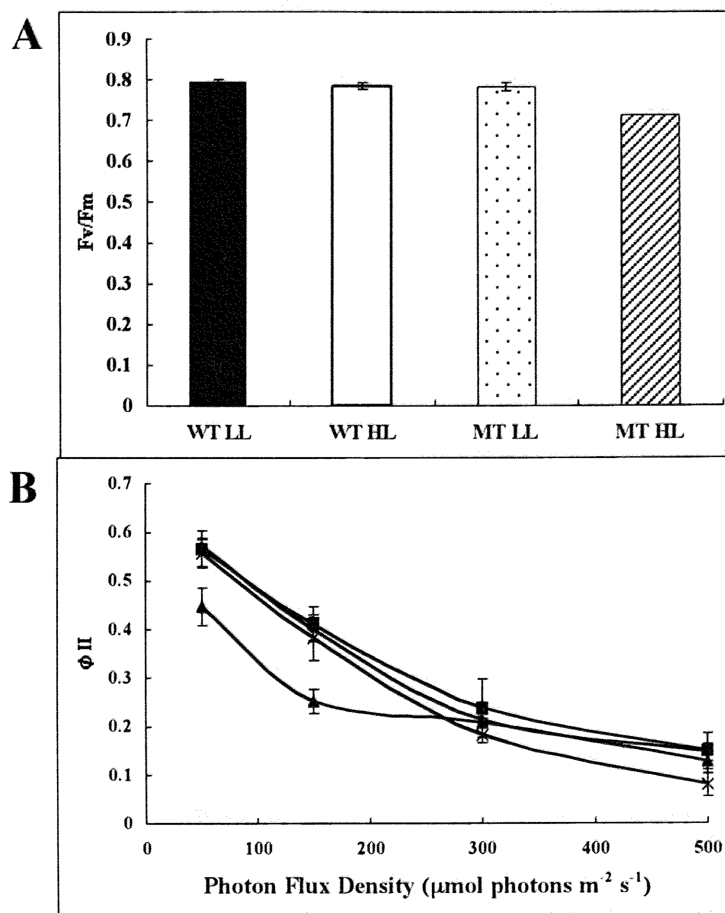


Figure 5. Analysis of chlorophyll fluorescence (Fv/Fm, φII)
Chlorophyll fluorescence of LL-acclimated and HL-acclimated plants (wild type and *ftsH6*) were measured by pulse amplitude modulation system (PAM), and each parameter (A; Fv/Fm, B; φII) was calculated (see Materials and method).The results are the means ± SD of three independent measurements. In the panel A, WT and MT represent wild type and mutant, respectively. In addition, LL and HL represent LL-acclimated and HL-acclimated plants, respectively.In the panel B, circle, square, cross, and triangle symbol represent WT LL, WT HL, MT LL, and MT HL, respectively.

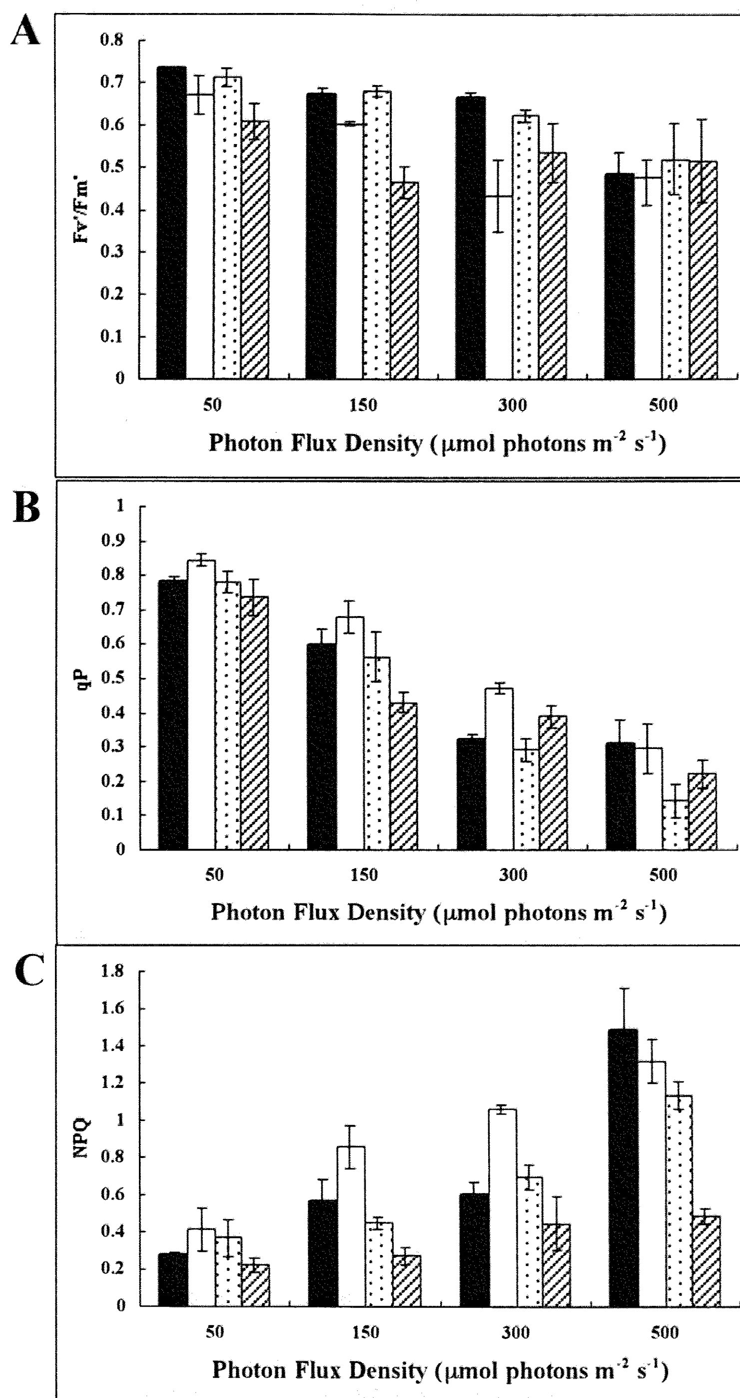


Figure 6. Analysis of chlorophyll fluorescence (Fv'/Fm', qP, NPQ)
Chlorophyll fluorescence of LL-acclimated and HL-acclimated plants (wild type and *ftsH6*) were measured using pulse amplitude modulation system (PAM), and each parameter (A; Fv'/Fm', B; qP, panel C; NPQ) is calculated (see Materials and method).The values are means of three independent measurements.Black, white, dotted, and striped bar correspond to WT LL, WT HL, MT LL, and MT HL, respectively.

32-11-13 mutant

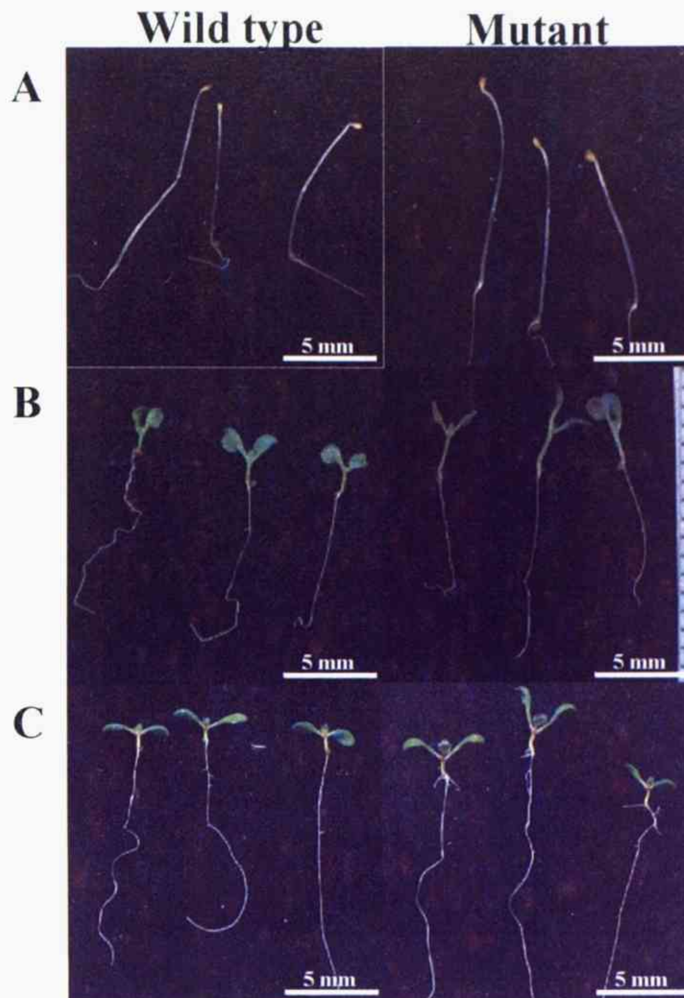


Figure 7. Phenotype of wild types and mutants at different photon flux density of growth light

Wild types and mutants (32-11-13) were grown for a week under several photon flux densities (A; Dark, B; 50 μmol photons m^{-2} s^{-1},C; 150 μmol photons m^{-2} s^{-1}). .Left panels and right panels are photographs of wild types and mutants, respectively.

32-11-13 mutant

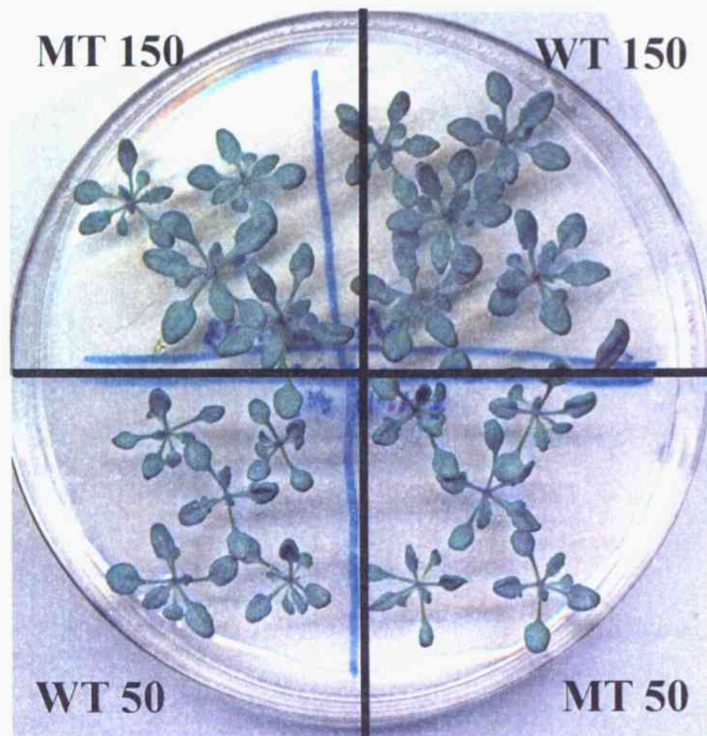


Figure 8. Growth of wild types and mutants at different photon flux density. Wild type plants (WT) and mutants (32-11-13; MT) were grown for 20 days under several light condition (lower panel; 50 μmol photons m^{-2} s^{-1}, upper panel; 150 μmol photons m^{-2} s^{-1}).

32-11-13 mutant

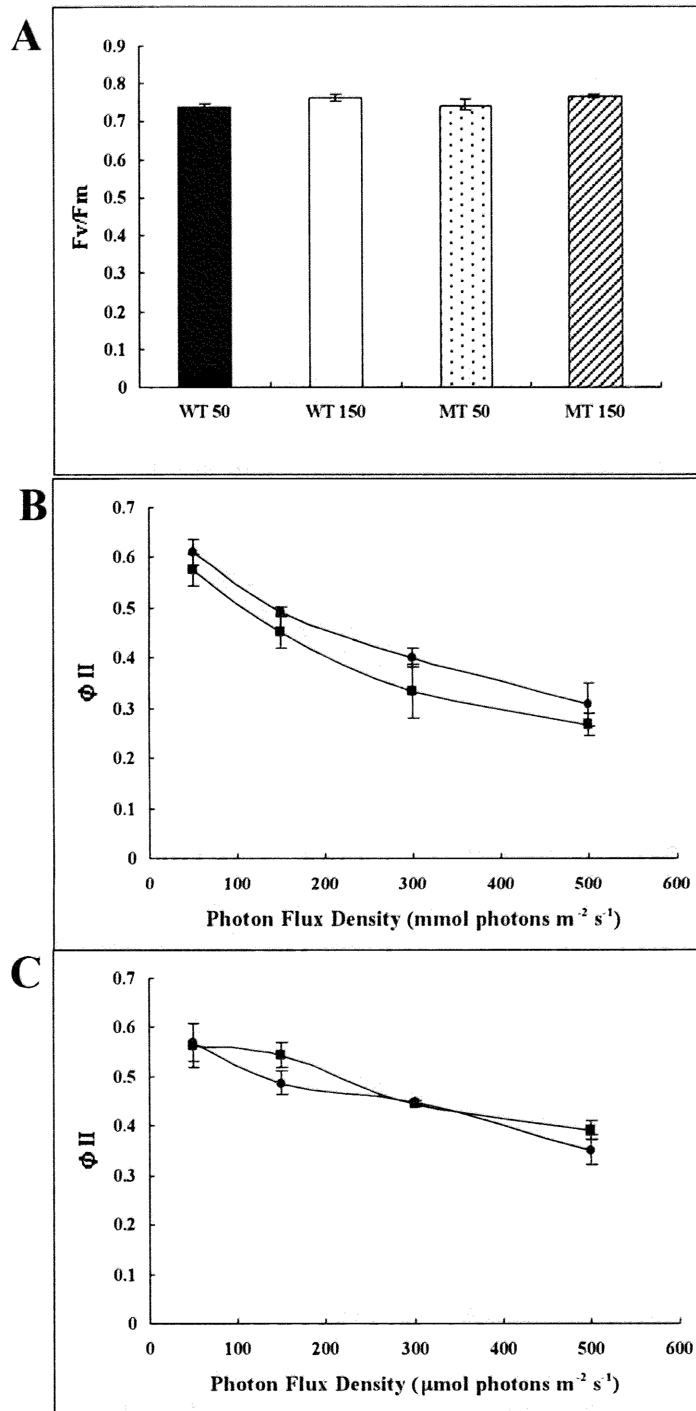
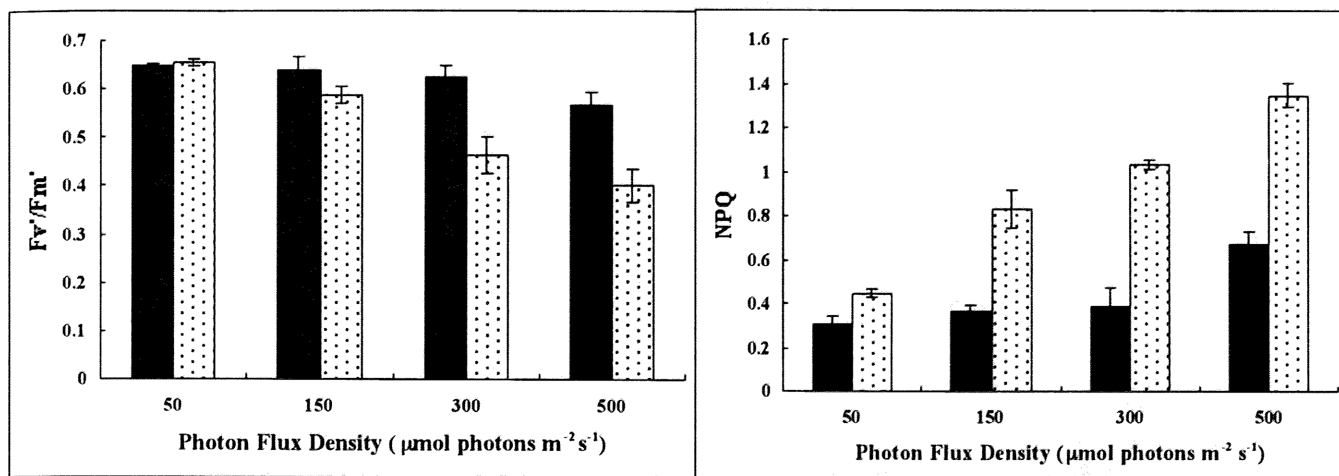


Figure 9. Comparison of chlorophyll fluorescence of wild types and mutants (Fv/Fm, ϕII)

Both wild types (WT) and mutants (32-11-13; MT) in Figure 8 are used for the measurement by PAM system, and each parameter (A; Fv/Fm, B; ϕII of 50 μmol photons m^{-2} s^{-1} grown plants, C; ϕII of 150 μmol photons m^{-2} s^{-1} grown plants) is calculated as described in Materials and method. The results are the means of three independent experiments. In panel A, 50 and 150 indicate the photon flux densities of the growth light.

32-11-13 mutant

A. 50 μmol photons m^{-2} s^{-1}



B. 150 **μmol photons m^{-2} s^{-1}**

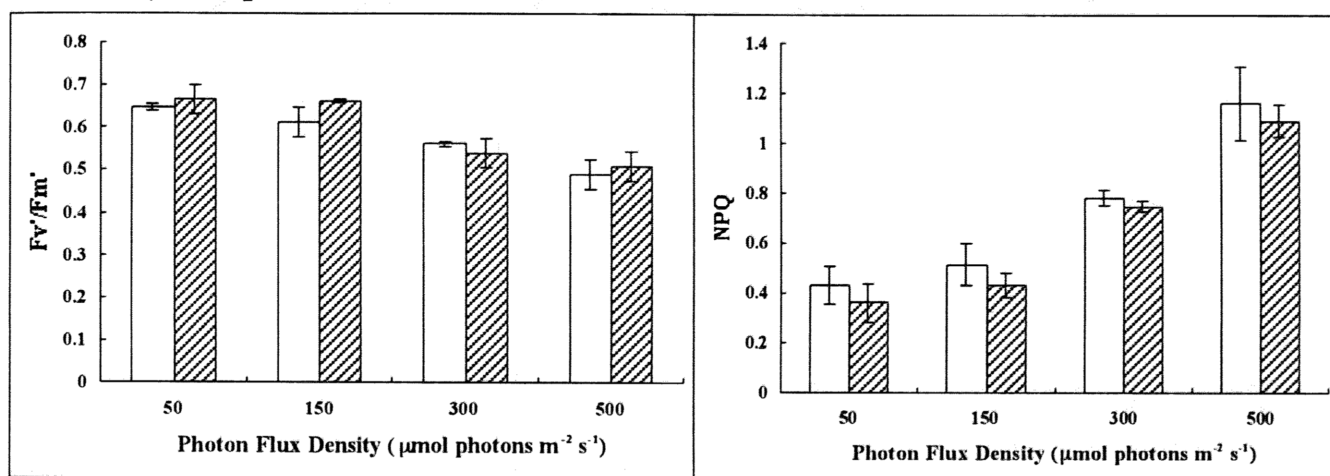
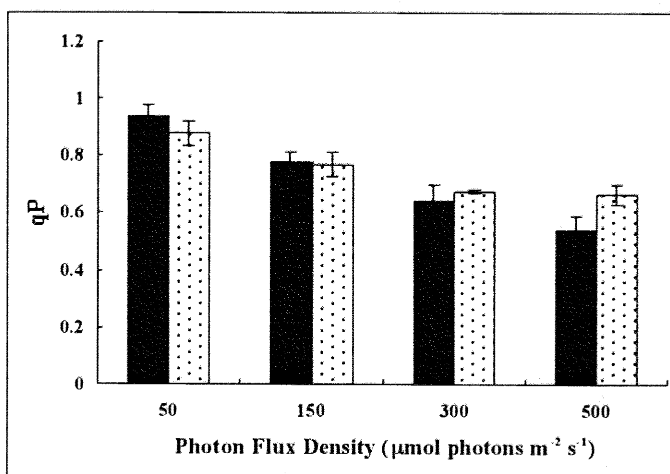


Figure 10. Comparison of chlorophyll fluorescence of wild types and mutants (Fv'/Fm', NPQ)

Both wild types (WT) and mutants (32-11-13; MT) in Figure 8 are used for the measurement by PAM system, and each parameter (A; Fv'/Fm' and NPQ of 50 μmol photons m^{-2} s^{-1} grown plants, B; Fv'/Fm' and NPQ of 150 μmol photons m^{-2} s^{-1} grown plants) is calculated as described in Materials and method. The results are the means of three independent experiments Black (A) and white (B) bars are for WT, dotted (A) and striped (B) bars are for MT.

32-11-13 mutant

A. 50 μmol photons m^{-2} s^{-1}



B. 150 **μmol photons m^{-2} s^{-1}**

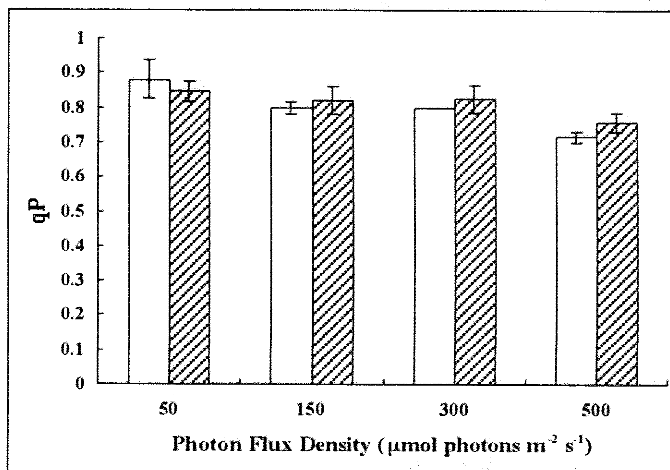


Figure 11. Comparison of chlorophyll fluorescence of wild type and mutant (qP)
Both wild types (WT) and mutants (32-11-13; MT) in Figure 8 are used for the measurement by PAM system, and each parameter (A; qP of 50 μmol photons m^{-2} s^{-1} grown plants, B; 150 μmol photons m^{-2} s^{-1} grown plants) is calculated as described in Materials and method. The results are the means of three independent experiments. The representation of the bars are same as in Figure 10.

Tables

		Chla(μg/FWmg)	Chlb(μg/FWmg)	Chla/Chlb
Wild type				
	LL	**1.69 ± 0.01**	**0.70 ± 0.02**	**2.44 ± 0.06**
	LL→HL	**0.84 ± 0.02**	**0.31 ± 0.01**	**2.74 ± 0.07**
	LL→LL	**1.50 ± 0.03**	**0.61 ± 0.01**	**2.47 ± 0.07**
Mutant				
	LL	**1.79 ± 0.04**	**0.71 ± 0.02**	**2.51 ± 0.02**
	LL→HL	**1.23 ± 0.01**	**0.50 ± 0.07**	**2.49 ± 0.08**
	LL→LL	**1.81 ± 0.03**	**0.73 ± 0.01**	**2.50 ± 0.02**

Table 1. Chlorophyll contents of wild types and mutants
Both wild types and *FtsH6* mutants were grown for 20 days under low light condition (LL; 50 μmol photons m^{-2} s^{-1}),and then transferred to high light condition (LL→HL; 300 μmol photons m^{-2} s^{-1}).Chlorophyll was extracted from a leave of each plant by DMF, and chlorophyll contents are determined spectrophotometreically. LL-acclimated plants are used as control (LL→LL).The results are the means of three independent experiments.

Photon Flux Density (μmol photons m^{-2} s^{-1})		Chla (μg/FWmg)	Chlb (μg/FWmg)	Chla/Chlb	Hypocotyl length (mm)
Dark					
	Wild type	n.d.	n.d.	n.d.	10.03 ± 1.99
	Mutant	n.d.	n.d.	n.d.	10.97 ± 1.86
50					
	Wild type	2.33 ± 0.17	0.86 ± 0.07	2.73 ± 0.02	0.97 ± 0.21
	Mutant	1.79 ± 0.14	0.65 ± 0.02	2.74 ± 0.13	1.93 ± 0.15
150					
	Wild type	1.18 ± 0.15	0.40 ± 0.05	2.95 ± 0.04	1.17 ± 0.06
	Mutant	1.20 ± 0.18	0.42 ± 0.07	2.88 ± 0.03	1.13 ± 0.15

Table 2. Chlorophyll contents and hypocotyl length of wild types and mutants
For determination of chlorophyll contents, both wild types and mutants (32-11-13) were grown for 17 days under several light conditions (Dark, 50, and 150 μmol photons m^{-2} s^{-1}).Chlorophyll was extracted from a leave of each plant by DMF, and chlorophyll contents are determined spectrophotometreically. For the measurement of hypocotyl length, plants were grown for 7days under the same photon flux densities as described above.The results are the averages of three independent measurements.