

PSB33 protein sustains Photosystem II in plant chloroplasts under UVA light

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Highlight

Plants utilize light for growth. As light differentiates during a day, month or year, it serves as a trigger for acclimation. Here we show that PSB33 is involved in a UVA light-mediated mechanism to sustain Photosystem II.

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Abstract

Plants can quickly and dynamically respond to spectral and intensity variations of the incident light. These responses include activation of developmental processes, morphological changes, and photosynthetic acclimation that ensure optimal energy conversion and minimal photoinhibition. Plant adaptation and acclimation to environmental changes have been extensively studied, but many details surrounding these processes remain elusive. The Photosystem II (PSII) associated protein PSB33 plays a fundamental role in sustaining PSII as well as in the regulation of the light antenna in fluctuating lights. We investigated how PSB33 knock-out plants perform under different light qualities. *psb33* plants displayed 88% lower fresh weight compared to wild type plants when cultivated in the border of UVA-blue light. The sensitivity towards UVA light was associated with a lower abundance of PSII proteins, which reduces *psb33* plants' capacity for photosynthesis. The UVA phenotype was further found to be linked to altered phytohormone status and changed thylakoid ultrastructure. Our results collectively show that PSB33 is involved in a UVA light-mediated mechanism to maintain a functional PSII pool in the chloroplast.

Key words

Arabidopsis, Blue light, Photoinhibition, State transition, Thylakoid membrane, UV light.

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Introduction

The absorbed light energy from the sun is not always beneficial for plants - in excess or under periods of environmental stress, the light causes inhibition of photosystem II (PSII) activity that may result in damage to the photosynthetic apparatus, a phenomenon referred to as photoinhibition (Kok, 1956; Li *et al.*, 2018). To minimize photoinhibition, plants have evolved protective mechanisms that ensure functional photosynthesis also under light stress conditions (Long *et al.*, 1994; Sarvikas *et al.*, 2006; Scheller and Haldrup, 2005; Yamamoto, 2016). These responses include limiting and dissipation of excess of absorbed light energy, and damage control by scavenging reactive oxygen species (ROS).

The thylakoid PSII auxiliary protein PHOTOSYSTEM II PROTEIN 33 (PSB33, AT1G71500) plays a specific role in the regulation of photoinhibition. PSB33 influences PSII-LHCII supercomplex organization in *Arabidopsis* (Fristedt *et al.*, 2015), and plants devoid of PSB33 display reduced state transition, i.e., ability to distribute excitation energy between the two photosystems (Fristedt *et al.*, 2017). Furthermore, *psb33* mutants show stunted growth in moderate, high, and fluctuating lights (Fristedt *et al.*, 2015; Fristedt *et al.*, 2017).

When we started cultivating *psb33* mutant plants in our new growth facility, we were unable to reproduce our previous finding that the mutant grows slower than wild type under standard conditions (Fristedt *et al.*, 2015). Instead of the expected 50% reduction in fresh weight compared to wild type, *psb33* mutants grew to almost similar size (Figure S1). We hypothesized that this variability in the size of *psb33* attributes to light spectral differences since the light intensity was identical between these two experiments.

Photoinhibition in plants is initiated by photoinactivation of PSII and followed by a repair cycle of the damaged subunits. There is currently no consensus regarding how photoinactivation of PSII is initiated and sensed by the plant. The two leading hypotheses, which are not mutually exclusive, are I) the acceptor-side PSII photoinhibition mechanism induced by singlet oxygen in excessive light, and II) the donor-side PSII photoinhibition occurring via light absorbed by the Mn_4O_5Ca cluster of PSII and occurring even in very low light intensities (Aro *et al.*, 1993; Zavafer *et al.*, 2015). However, from studies on the action spectrum of PSII photoinactivation, it is evident that the extent of photoinhibition is wavelength dependent and that light in the UV region of the spectrum (100-400 nm) causes

the strongest photoinactivation of PSII (Jones and Kok, 1966; Sarvikas *et al.*, 2006; Takahashi *et al.*, 2010), predominantly targeting the Mn₄O₅Ca cluster of the reaction center and the acceptor side of PSII (Hakala *et al.*, 2005; Ohnishi *et al.*, 2005). Thus, non-photochemistry wavelengths can substantially affect PSII photoinhibition.

The relative excitation pressure on the two photosystems, PSI and PSII, changes depending on light intensity and spectral properties, and the inability to appropriately distribute the light energy between the photosystems may lead to photoinhibition (Bellafiore *et al.*, 2005). Plants can reallocate a fraction of the light-harvesting antenna complex II (LHCII) between PSI and PSII through a process known as state transition (Adamska *et al.*, 1992; Allen *et al.*, 1981). The phosphorylation of the LHCII antenna proteins turns on this reallocation. (Mekala *et al.*, 2015). High excitation pressure on PSII activates the serine-threonine kinase STN7 that phosphorylates LHCII proteins (Bellafiore *et al.*, 2005; Fristedt and Vener, 2011; Trotta *et al.*, 2016). This results in enhanced delivery of excitation energy from LHCII to PSI, thus increasing its light-harvesting capacity (State II). Over-excitation of PSI, on the other hand, triggers the dephosphorylation of LHCII by the PPH1/TAP38 phosphatase (Pribil *et al.*, 2010; Shapiguzov *et al.*, 2010). Dephosphorylated LHCII associates with PSII to increase its capacity to absorb energy (State I). Under artificial illumination, State I is induced by low-intensity light (Rintamäki *et al.*, 2000) as well as red (636-700nm) and blue (400-500 nm) light (Bellafiore *et al.*, 2005; Trotta *et al.*, 2016). Far-red light (700-780 nm) preferentially excites PSI and triggers State II (Adamska *et al.*, 1992; Bonardi *et al.*, 2005).

Repair of photodamaged PSII proteins is efficient and under strict quality control, especially of the D1 subunit. This photoinhibition/repair cycle follows a sequential series of highly regulated steps: monomerization of damaged PSII dimers, migration of PSII monomers from appressed to unappressed thylakoid regions, degradation of the damaged D1 protein by FtsH protease, and finally, *de novo* production of D1 subunits insertion into PSII monomers (Ohad *et al.*, 1984; Yoshioka-Nishimura and Yamamoto, 2014). Phosphorylation of the PSII core proteins by the STN8 kinase is important for the repair cycle and influences the disassembly of the damaged PSII dimer and protects D1 protein from excess degradation (Fristedt *et al.*, 2009; Kato and Sakamoto, 2014; Tikkanen *et al.*, 2008).

Apart from directly fueling photochemistry, light of different colors and intensities serves as cues during plant development and growth. A large number of photoreceptors allow plants to sense light over a broad spectrum of wavelengths, from UV-B (280-320 nm) to far-red. Phytochromes absorb red and far-red light in the 600-750 nm region and play central roles in plant germination, de-etiolation, stomata development, flowering, shade avoidance, and senescence (Wang and Wang, 2014). Blue light and UV light are perceived and effectively transduced as a signal by the photoreceptor families cryptochromes (400-500 nm), phototropins (320-500 nm), zeitlupes (450-520 nm) and UV-B photoreceptors (280-320 nm) (Christie *et al.*, 2015). These photoreceptors control processes that include photomorphogenesis, flowering, circadian period, phototropism, and stomata opening. Signaling pathways downstream of the photoreceptors often integrate with hormone networks and influence their levels (Chaiwanon *et al.*, 2016). Although well studied, there is limited evidence linking photoreceptors directly to photosynthesis regulation. Photoprotective mechanisms identified in green algae and cyanobacteria have been tied to UV and or blue light perception by photoreceptors (Allorent *et al.*, 2016; Kirilovsky and Kerfeld, 2013; Petroutsos *et al.*, 2016). Further, blue light perception in diatoms increases their photoprotective potential (Schellenberger Costa *et al.*, 2013). However, these are aquatic organisms exposed to an environment where blue light dominates. In land plants, phototropins activated by blue light stimulate the relocation of chloroplast (Goh, 2009). This process acts to distribute PSII damage among the different cell layers of the leaf in high light (Cazzaniga *et al.*, 2013). More indirectly, blue and UVA (320-400 nm) light play a role in the regulation of the PSII complex as they stimulate the expression of genes encoding Light-harvesting chlorophyll a/b-binding proteins (Lhcbs) (Folta and Kaufman, 1999) and a special class of PSII core proteins (*PSBD-PSBC*, D2, and CP43) (Christopher and Mullet, 1994).

Here, we aimed to investigate if changes in light quality affect growth and photosynthetic responses in *psb33* mutant plants. We found that the *psb33* plants show a conditional dwarf phenotype when cultivated under blue light, specifically in UVA or near UVA light. This UVA light-dependent phenotype was found to be associated with low levels of PSII proteins, abnormal thylakoid ultrastructure, and changed phytohormone levels. We suggest that PSB33 is involved in a UV light-mediated mechanism to maintain an efficient PSII repair by regulating the *de novo* synthesis of PSII.

Material and Methods

Plant material and growth conditions

Arabidopsis thaliana seeds were sown on soil and vernalized at 4°C for two days before transfer to climate-controlled growth chambers (CLF PlantMaster, Plant Climatics, Wertingen, Germany). The plants were grown under a 12h photoperiod at 20°C/18°C light/dark in 60% relative humidity. Seedlings were transplanted into individual pots after ten days and moved to chambers with LED lamps after an additional four days. All mutants and wild type used in the study were in the Columbia-0 genetic background, previously described for *psb33-3* (Fristedt *et al.*, 2015), *stn7* (Bellafiore *et al.*, 2005), *stn8* and *stn7stn8* (Bonardi *et al.*, 2005), *tap38* (Pribil *et al.*, 2010), *amiLhcb1* and *amiLhcb2* (Pietrzykowska *et al.*, 2014), *psbw* (García-Cerdán *et al.*, 2011), *psal* (Lunde *et al.*, 2000), *pgr5* (Munekage *et al.*, 2002) and *npq4* (Johnson and Ruban, 2010). The LED lights were provided by Heliospectra L4A lamps (Heliospectra, Gothenburg, Sweden) with diode peak wavelengths at 400, 420, 450, 530, 630, 660, and 735 nm. For the experiment with the 385 nm LEDs, Heliospectra RX30 lamps (Heliospectra, Gothenburg, Sweden) were used. Full light spectra were recorded with a JAZ spectrometer (Ocean optics, Dunedin, FL, USA). Lamps were set up to ensure an even light distribution of the different colors. For example, when blue and red light were combined, 50% of the total photon flux was provided by blue LEDs and 50% by red LEDs.

Pigment content, hormonal analysis, and chlorophyll a fluorescence

Chlorophyll and carotenoids were extracted from leaf discs in 95% (v/v) ethanol for 10 min at 60°C and concentrations were determined spectrophotometrically (Lichtenthaler and Wellburn, 1983). Plant hormone levels were determined from leaf discs (12 mm diameter) as described (Flokova *et al.*, 2014).

Chlorophyll *a* fluorescence parameters were recorded with a Pocket PEA Chlorophyll Fluorimeter (Hansatech Instruments Ltd, King's Lynn, Norfolk, UK). The maximum quantum efficiency of Photosystem II (F_v/F_m , calculated as $(F_m - F_0)/F_m$) and performance index (PI_{ABS}) (Strasser *et al.*, 2004) were determined in attached leaves dark-acclimated for 20 min in room temperature with 1 s measuring light with the intensity of 3500 $\mu\text{mol m}^{-2}\text{s}^{-1}$.

Thylakoid membrane preparation, SDS-PAGE and immunoblotting

For extraction of whole leaf proteins, tissue (approximately 200 mg) was frozen in liquid nitrogen, grounded into a fine powder, mixed with buffer (100 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0, 250 mM NaCl, 0.75% SDS (v/v), 1 mM DTT, 10 mM NaF, 1X Protease inhibitor cocktail set I (Calbiochem, Merck KGaA, Darmstadt, Germany)) and denatured at 68°C for 10 minutes. Centrifugation removed cellular debris, and chlorophyll content was determined as described (Porra *et al.*, 1989). Proteins were loaded on 5%/14% stacking/separating acrylamide gels based on chlorophyll content (100-500 ng μl^{-1} depending on the expected abundance of the protein). SDS-PAGE separation, protein transfer to PDVF membranes, and detection were as described (Fristedt *et al.*, 2015). Antibodies used in this work were all from Agrisera Antibodies (Vännäs, Sweden): PsaB, PsaA, RbscL, PsbA, PsbD, Lhcb2-P (AS13 2705), Lhcb2 (AS01 003), Lhcb1-P (AS13 2704), Lhcb1 (AS01 004).

Staining for reactive oxygen species

Staining for H_2O_2 was performed as previously described with minor modifications (Daudi and O'Brien, 2012). Briefly, seedlings were vacuum inoculated in 10 mM Na_2HPO_4 3,3'-diaminobenzidine (Saadatian-Elahi *et al.*) staining solution at approximately 100 mbar for 2 min. After two hours in darkness, the seedlings were destained in 96% (v/v) ethanol overnight and photographed. Staining for superoxide radicals was performed similarly but using 0.1% nitroblue tetrazolium in 50 mM Na_2HPO_4 (pH ~7).

Transmission electron microscopy

Leaves from plants grown in fluorescent white, red, or blue light were collected two hours after the onset of light following a 14h night period. Tissue preparation and imaging were as described (Herdean *et al.*, 2016).

PSII electron transfer properties

Electron transfer properties in PSII were measured by flash-induced variable fluorescence, thermoluminescence, and low-temperature electron paramagnetic resonance spectroscopy (EPR) in thylakoid membranes isolated from wild type and *psb33* mutant plants. Fluorescence decay kinetics were measured using an FL3000 dual modulation kinetic fluorometer, and thermoluminescence signal was measured using a TL200/PMT

thermoluminescence system (both from Photon System Instruments, Brno, Czech Republic) as described in (Volgusheva *et al.*, 2016).

Low-temperature EPR was measured in X-band ELEXSYS 500 spectrometer using a SuperX EPR049 microwave bridge and a Bruker 4122SHQE super high-Q cavity (Bruker BioSpin). The system was fitted with a 900-cryostat and an ITC-503 temperature controller from Oxford instruments Ltd. The S₂ state multiline signal was induced by illumination at 200 K for 6 min, and full oxidation of Cytochrome b₅₅₉ was induced by illumination at 77 K as described in (Chen *et al.*, 2011). Processing and analysis of EPR spectra were performed in Bruker Xep program.

Statistical analyses

Data was handled in Microsoft Excel 2010 (Microsoft, USA) with the add-in Daniel's XL Toolbox (<https://www.xltoolbox.net/>) or in IBM SPSS Statistics version 25 (IBM corporation, Armonk, US). The normality of data was tested using the Shapiro-Wilk method. Non-normally distributed data were log-transformed before parametric tests were performed. Results were considered significant if two-tailed $p < 0.05$.

Results

psb33 mutant plants show a conditional light quality phenotype

We set out to investigate if the lack of a slow-growth phenotype of *psb33* in our new plant growth facility might be attributed to specific qualitative properties of the fluorescent lights in these chambers (Figure S1). Therefore, plants were grown under fluorescent white light at a total photon flux density of 120 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (PFD) for two weeks and then transferred to blue [400,420,440] nm, red [630,660] nm, or a combination of blue and red LED lights, for additional three weeks (Figure 1). Wild type and control plants were maintained in the fluorescent white light throughout the growth period. The total light intensity was kept constant at 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in all different spectral conditions (Figure 1, left panel). To test if the growth phenotype of *psb33* was associated with its inability to induce state transition, the double mutant *stn7stn8* was included as a negative control in the experiment (Tikkanen *et al.*, 2010). After two weeks of growth under fluorescent white

light, before transfer to LED lights, there was no apparent difference in size between the wildtype, *psb33* and *stn7stn8* (Figure S2).

The *stn7stn8* plants displayed an approximately 20% reduction in fresh weight compared to wild type after 5 weeks in all light conditions except in red light where they grew to a similar size (Figure 1). *psb33* plants, on the other hand, showed similar fresh weight as wild type in fluorescent white and red light while a dramatic and statistically significant (one-way ANOVA, $p < 0.001$) decrease in blue light was observed. The fresh weight of the *psb33* plants grown under a combination of red and blue light displayed an intermediate reduction in size compared to those cultivated in white or blue light. The growth phenotype of *psb33* in the blue light did not correlate with changes in chlorophyll or carotenoid content (Table 1).

These data point to the fact that the slow-growth phenotype of the *psb33* mutant is related to sensitivity towards blue light. Further, the phenotype appeared not to be a consequence of impaired state transition, since plants lacking the kinases STN7 and STN8 (*stn7stn8* plants) did not respond similarly as *psb33* to the tested light regimes.

Chlorophyll fluorescence characteristics of psb33 in blue and red light

Next, we determined the photosynthetic performance of plants grown under white, blue, and red or a combination of blue and red light (Figure 2A). The maximum quantum efficiency of Photosystem II (F_v/F_m) was similar between the control plants (wild type and the *stn7stn8*) and showed only small variations in the different light conditions. Following our previous results (Fristedt *et al.*, 2015), F_v/F_m in *psb33* was slightly, yet statistically (one-way ANOVA, $p < 0.001$) lower than in wild type in white light. However, in blue, red, and combined blue/red light, the F_v/F_m values were reduced by approximately 20% in *psb33*, indicating decreased PSII efficiency in these conditions (Figure 2A). In repeated experiments, F_v/F_m was consistently lower in *psb33* plants grown in blue light compared to plants grown in red light. However, this difference was not statistically significant in all experiments (data not shown). The performance index parameter (PI_{ABS}) is an overall parameter of plant vitality and a sensitive index to evaluate stress (Kalaji *et al.*, 2014). PI_{ABS} takes into account the concentration of active reaction centers, the force of the light reactions, as well as the force of the dark reactions (Strasser *et al.*, 2000). Although there were some variability

between replicates, *stn7stn8* plants largely displayed similar PI_{ABS} as the wild type in all light conditions (Figure S3). However, *psb33* plants grown in blue, red, and the combination of blue and red lights displayed a decrease in PI_{ABS} in comparison to *psb33* plants grown in white fluorescence light.

To further explore the response of *psb33* to blue and red light. Plants grown in fluorescent white light were transferred to blue and red light and Fv/Fm was determined at intervals for up to three days (Figure 2B and C). Recovery of the plants was assessed by monitoring the chlorophyll fluorescence after the transfer of plants back to white light. Both blue and red light-induced a fast decrease of the Fv/Fm ratio and reached similar values as in the long term acclimated plants after three days. Following three days of recovery in white light, Fv/Fm completely reverted to steady-state values again, showing that these effects were reversible (Figure 2B and C).

The Chl fluorescence phenotype of psb33 is not found in other related photosynthetic mutants

To gain insight into the mechanism behind the strong response of the *psb33* plants to blue and red light, we compared the performance of *psb33* under these light treatments to that of well-characterized photosynthesis mutants. Specifically, mutants lacking components of the photosynthetic machinery associated with excitation energy transfer were selected and compared to *psb33* (Figure 2D and E). The kinases STN7 and STN8, and phosphatase TAP38 regulate the phosphorylation status of PSII core and LHCII proteins (Bellafiore *et al.*, 2005; Bonardi *et al.*, 2005; Pribil *et al.*, 2010; Shapiguzov *et al.*, 2010). The *stn7*, the *stn8*, the *stn7stn8*, and the *tap38* mutants have reduced state transition capacity and differ in sensitivity to high or fluctuating light (Fristedt *et al.*, 2009; Tikkanen *et al.*, 2010). The *lhcb1* and the *lhcb2* mutants, corresponding to plants deficient in the Lhcb1 and the Lhcb2, respectively, show altered antenna and thylakoid structure, impaired state transition, and reduced NPQ (Pietrzykowska *et al.*, 2014). The *npq4* mutant is devoid of the protein PsbS and lacks the qE component of NPQ, is sensitive to photoinhibition (Li *et al.*, 2002) and fluctuating light (Külheim *et al.*, 2002). PsbS has also been proposed to play a role in energy distribution between the two photosystems (Jajoo *et al.*, 2014). PsbW plays an important

role in PSII function, and the corresponding *psbw* mutant shows destabilized PSII supercomplex organization, decreased phosphorylation of PSII core proteins, and faster changes of the redox state of the plastoquinone pool (García-Cerdán *et al.*, 2011). *psal* mutants lack a PSI component, show reduced PSI electron transport rate, normal phosphorylation of LHCII but inability to increase the PSI antenna size in State II light (Lunde *et al.*, 2000). Finally, PGR5 encodes a protein previously thought to be involved in cyclic electron transport around PSI (Munekage *et al.*, 2002), but more recently shown to be involved in regulating proton flux across the thylakoid membrane (Kanazawa *et al.*, 2017; Suorsa *et al.*, 2012). The *pgr5* mutant is unable to induce NPQ, is sensitive to high light, which results in PSI damage (Munekage *et al.*, 2002), shows stunted growth in normal conditions, and is unable to cope with fluctuating light resulting in wilting (Tikkanen *et al.*, 2010).

Comparing the *Fv/Fm* parameter of all these mutant lines and wild types in response to blue and red light, it is clear that the *psb33* mutant was the only line showing a substantial reduction in photosynthetic performance (Figure 2D and E). There was no statistical difference in *Fv/Fm* among the *psb33* plants kept for three or seven days in blue or red light. These results indicated that the observed phenotype of *psb33* is not connected to thylakoid Δ pH-dependent regulation mechanisms since *pgr5* and *npq4* were unaffected under the tested light regimes. The results further strengthened the notion that the phenotype of *psb33* cannot be explained by the lack of photosystem energy distribution-dependent mechanisms since none of the included mutants with impaired ability to phosphorylate PSII core or with altered antenna proteins displayed stress symptoms under red or blue light.

psb33 plants display altered phytohormone levels in blue and red light

Plant growth restrictions are commonly associated with defective hormone control. Therefore, we tested if the response of *psb33* plants to blue and red light was accompanied by changes in leaf phytohormone levels (Figure 3). Our analysis specifically targeted hormones involved in growth and stress signaling: jasmonates (including JA, isoleucine-conjugated JA (JA-Ile), and 12-oxo-phytodienoic acid (*cis*-OPDA)), salicylic acid (SA), indole acetic acid (IAA), and IAA derivatives. SA levels were 3.6 and 5.5 times lower ($p < 0.01$, one-

way ANOVA, Bonferroni-Holm posthoc test) in the mutant compared to wild type in blue and red light, respectively. However, levels were relatively low in both the wild type and *psb33* in all conditions (Figure 3F). The only statistically significant hormonal change exclusively apparent in *psb33* grown in blue light was a strong accumulation of aspartic acid conjugated IAA (IAA-Asp) (Figure 3C).

Blue light induces changes to psb33 thylakoid ultrastructure

To further explore the *psb33* growth phenotype and to resolve whether it relates to morphological changes at the microscopic level, the thylakoid ultrastructure of *psb33* plants grown in white, blue, and red light was examined by transmission electron microscopy (Figure 4). No difference in thylakoid structure was observed between wild type and *psb33* plants grown in fluorescent white- or red light. Thylakoids of *psb33* grown in blue light were found to be swollen, showing large luminal spaces at non-appressed regions of the thylakoid membrane (Figure 4B). Such swollen thylakoids could be observed in a few chloroplasts also from wild type plants taken from blue light. However, virtually all thylakoids from *psb33* plants from blue light displayed such abnormal structures.

UV-A light causes the psb33 growth phenotype

Next, we set out to more specifically delineate the light wavelengths causing the deleterious growth phenotype of *psb33* in blue light. Three blue light conditions provided by three different LEDs were tested at the PFD of $120 \mu\text{mol m}^{-2} \text{s}^{-1}$: [400], [420,440], and [400,420,440] nm (Figure 5). *psb33* plants cultivated under only 400 nm light only reached 12% of wild type fresh weight at the end of the growth period (Figure 5A and B), while *psb33* plants in [420,440] nm and [400,420,450] nm reached 66% and 40%, respectively, of the weight of wild type.

Due to the fact that the 400 nm LED lamp emits light ranging from 391 nm to 421 nm, with a peak at 404-405 nm, we suspect that light in the UVA region is the main cause of the dwarf phenotype of *psb33* grown in blue light (Figure 1, Figure 5, Figure S4). Growing plants in 385 nm UVA light corroborated this finding: *psb33* plants displayed strong growth restriction

compared to the wild type in this light condition (Figure S5). Chlorophyll *a* fluorescence measurements showed a drastic reduction in quantum efficiency (*Fv/Fm*) of PSII and PI_{ABS} in *psb33* plants grown in lights with UVA (Figure 5C and S5). The *Fv/Fm* ratio of *psb33* plants grown in [420,450] nm was significantly higher (one-way ANOVA, $p < 0.01$) than in plants grown in [400] nm or [400,420,440] nm (Figure 5C). Thus, plants cultivated in light conditions lacking UVA wavelengths performed better in terms of photosynthetic performance than plants grown in lights containing UVA.

PSII core protein levels are reduced in psb33

The low *Fv/Fm* measured in the *psb33* mutant indicated decreased PSII efficiency and possibly damage to the PSII complex. We, therefore, examined potential photodamage to PSII in *psb33* grown in various blue light conditions. To investigate if PSII and PSI protein levels were influenced by the quality of light, whole-leaf proteins were extracted from plants grown in either fluorescent white light, blue light with or without the 400 nm UVA component (Figure S4) ([400,420,440] nm or [420,440] nm respectively), and red light. Further, the fresh weight of plants was determined, and whole leaf proteins extracted from wild type and *psb33* plants grown under blue and red light in combination with far-red light ([400,20,440,730] nm or [630,660,730] nm, respectively) (Figure S7). Far-red light was included in the experimental setup since it predominantly excites PSI. The far-red light treatment could thus reveal if increased excitation pressure of PSI would balance the relative energy distribution between the two photosystems and reduce photodamage. Extracted whole leaf proteins were separated on SDS-PAGE, immunoblotted and labeled with antibodies specific for PSII (D1 and D2), PSI (PsaA and PsaB), LHCII (Lhcb1 and Lhcb2) and its phosphorylated forms (Lhcb1-P and Lhcb2-P) and Rubisco large subunit (RbcL) (Figure 6A). PsaA and PsaB levels varied in the different light conditions but were largely similar between wild type and *psb33*. So was the abundance of RbcL. In agreement with the impaired state transition phenotype of *psb33*, phosphorylation of Lhcb1 and Lhcb2 was almost completely lost in the *psb33* mutant in blue light. Notably, in red light and light supplemented with far-red, Lhcbs were phosphorylated to a certain level in *psb33* plants.

Furthermore, very little phosphorylation of Lhcb proteins was observed in *psb33* in fluorescent white light, corroborating our previous findings (Fristedt *et al.*, 2017). Our compiled data thus indicated that the redistribution of energy between the two photosystems through re-arrangement of the LHCII light-harvesting antenna is impaired in *psb33*. Yet, this deficiency cannot alone explain the blue light growth phenotype. The PSII core proteins D1 and D2 were both reduced in *psb33* in blue light conditions. The corresponding reduction in protein abundance occurred in *psb33* exposed to 400 nm LED light, whereas plants exposed to [420,440] nm light displayed higher levels of D1 than plants from only [400] nm or [400/420/440] nm (Figure 6A and B).

PSII electron transport properties are modified in psb33

The reduced levels of the D1 and D2 proteins in *psb33* cultivated under UVA light may indicate an altered redox state of the acceptor side of PSII. To investigate this, we performed flash-induced variable fluorescence decay and thermoluminescence measurements in isolated PSII membranes from wild type and *psb33* plants. Analysis of the flash-induced fluorescence decay kinetics demonstrated faster electron transfer from Q_A^- to Q_B in *psb33* mutant (Figure 7A, left panel) (Mamedov *et al.*, 2000; Roose *et al.*, 2010; Vass *et al.*, 1999). Also, thermoluminescence measurements, which are a useful complement to the flash-induced fluorescence decay measurements (Ducruet and Vass, 2009; Vass and Govindjee, 1996), also showed the presence of only a slightly up-shifted B-band (which represents the $Q_B^- \rightarrow S_2$ state recombination) at 38 °C. This is different compared to a mixture of the Q-band (which represents the $Q_A^- \rightarrow S_2$ state recombination) and B-band, present in thylakoids from wild type at 14 °C and 36 °C (Figure 7B, left panel). Thus, both measurements confirm the presence of more oxidized acceptor side of PSII and higher redox potential of Q_B in *psb33* mutant.

The fluorescence decay kinetics and thermoluminescence bands measured in the presence of DCMU (and in case of thermoluminescence indicating only the $Q_A^- \rightarrow S_2$ state recombination), were virtually the same in the wild type and *psb33* mutant (Figure 7A and B, respectively, right panel). This indicated that the redox properties of Q_A and the water oxidizing complex on the donor side of PSII were not affected in the *psb33* mutant. This was

also corroborated by EPR measurements of the S_2 state multiline signal which were found similar in wild type and *psb33* mutant (Figure 7D).

Interestingly, Cytochrome b_{559} , an integral part and an alternative electron donor/acceptor of the PSII reaction center (Shinopoulos and Brudvig, 2012) was found to be predominantly in the low potential form in the *psb33* mutant (Figure 7E) again, pointing out the more oxidized acceptor side in PSII. However, the overall amount of the PSII reaction centers, as judged from the Tyrosine D radical on the Chl basis, was only slightly diminished in the *psb33* mutant (Figure 7C).

psb33 ROS levels are unaffected by UVA light

The redox potential of Q_B is important for the regulation of electron transfer both for forward and backward electron transfer, which functions as a protective mechanism for PSII (Kato *et al.*, 2016). The modulated potential of Q_B in combination with elevated excitation pressure on PSII via UVA light, could induce ROS mediated damage to D1 and D2 in *psb33* mutant plants. To test this, we investigated if *psb33* mutant plants differed in ROS levels compared to wild type plants in UVA light. Plants grown in [400] or [420,450] nm light were stained for hydrogen peroxide using 3,3'-diaminobenzidine and superoxide ($O_2^{\cdot-}$) radicals using nitroblue tetrazolium (NBT). Surprisingly, no visual differences in levels of either H_2O_2 by DAB or $O_2^{\cdot-}$ by NBT were observed between wild type and *psb33* (Figure 8).

Discussion

The mechanisms by which plants maintain and optimize photosynthesis in fluctuating light environments are rapidly being unraveled (Cruz *et al.*, 2016; Tikkanen *et al.*, 2010; Tikkanen *et al.*, 2012; Vialet-Chabrand *et al.*, 2017). But as natural light fluctuates in intensity, it also varies considerably in quality, and the influence of light color in photosynthetic regulation has so far largely been overlooked (Allorent and Petroustos, 2017). In this study, we demonstrate that plants lacking PSB33 are particularly sensitive to light in the UVA spectral region. UVA light was found to induce a dwarf phenotype in *psb33* plants, which was associated with diminished PSII protein levels.

PSB33 has a specific spectral influence on PSII and linear electron flow

Since the growth phenotype of the *psb33* mutant appeared to be very variable and strictly dependent on light conditions in our different growth facilities, we decided to pay particular emphasis on the light quality in inducing the *psb33* phenotype in this work. By cultivating the mutant in different light settings, it was found that the reduced growth of *psb33* was specifically induced by blue light (Figure 1 and S3), particularly the spectral region that composes the UVA (Figure 5). We, therefore, suggest that the previously reported growth phenotype, reaching approximately 50% the size of the wild type under normal growth condition (Fristedt *et al.*, 2015), was due to the presence of short-wavelength blue light in the light source of the growth chambers.

The slow-growth of *psb33* in blue light was found to be connected to low levels of PSII core proteins D1 and D2 (Figure 6) which inevitably leads to reduced photosynthesis rate. The effect was more pronounced in UVA (400 nm) blue light than in [420,450] nm or [400,420,450] nm blue light at the same total photon flux (Figure 6B). The red light did not cause any difference in D1 or D2 protein abundance between wild type and mutant. It is interesting to note that both blue- and red light-grown plants displayed a similar reduction in maximal PSII quantum yield (F_v/F_m) (Figure 2) and PI_{ABS} (Figure S3). However, *psb33* plants grown in exclusively blue light at 400 nm showed further reduced F_v/F_m values (Figure 5C).

Supplementing UVA containing blue light with far-red light rescued the phenotype to some extent both in terms of growth and PSII protein levels (Figure 6A and Figure S7). As far-red light predominately excites PSI, a possible explanation to this rescue is that PSB33 has a role in the linear electron transport chain to balance the redox components between the two photosystems. In the absence of PSB33, an unbalance of redox components in the electron transport chain would initiate the production of ROS, resulting in elevated PSII damage. However, we did not observe an increase of ROS in leaves from plants grown in UVA light that would support an over-excitation of PSII and subsequently increased photodamage. Another, perhaps more likely explanation is that the rescue effect of far-red is a consequence of reduced UVA blue light as the total photon flux was kept constant in the experiments (i.e., 50% of the total light was provided by far-red and 50% by blue light).

When we tested mutants with reported impaired ability for PSII super-structure (*psbw*), state transitions (*lhcb1*, *lhcb2*, *psal*, and *stn7/8*), cyclic electron transport and proton gradient (*pgr5*) or NPQ (*npq4*), none exhibited a slow-growth phenotype comparable to that of *psb33* in blue light, indicating that PSB33 has a unique role in blue light perception unrelated to these known pathways to maintain photosynthesis.

Salicylic acid and aspartic acid conjugated indole-3-acetic acid hormone levels differ in psb33 plants exposed to different lights

SA levels are well known to influence plant growth negatively and to be important for light acclimation (Rivas-San Vicente and Plasencia, 2011). Surprisingly, the SA level decreased in response to both blue and red light treatments in *psb33*, whereas it increased in the wild-type (Fig. 3F). SA and glutathione interplay to regulate antioxidant response through ROS scavenging (Herrera-Vasquez *et al.*, 2015). The low levels of SA in *psb33* could accordingly explain the accumulation of different ROS in the mutant line, as previously reported (Fristedt *et al.*, 2015). However, we could not observe any differences between wild type and *psb33* in, or the absence of, UVA light ([400] nm and [420,450] nm, respectively) in terms of H₂O₂ and superoxide radicals (Fig. 8), suggesting that changed SA and ROS levels did not contribute to the deleterious effect of UVA light. The only hormonal change exclusively observed in *psb33* grown in blue light was a strong accumulation of aspartic acid conjugated IAA (IAA-Asp) (Figure 3C). This is believed to be an inactive form of IAA that is destined for catabolism (Ludwig-Muller, 2011; Woodward and Bartel, 2005).

Psb33 plants grown in blue light show an abnormal thylakoid ultrastructure.

PSB33 is primarily located in non-appressed thylakoid regions (Fristedt *et al.*, 2015; Fristedt *et al.*, 2017; Kato *et al.*, 2017). Quality control of PSII is dependent on reversible thylakoid structure dynamics (Yamamoto, 2016). Light induces a reversible swelling and unstacking of the thylakoids, which are necessary for proper PSII repair and protects PSII from damage (Khatoon *et al.*, 2009). Furthermore, the increased surface area of the membranes influences its fluidity, important for lateral movement of proteins and other compounds

within the membrane regulating NPQ, state transition, and PSII repair (Yamamoto, 2016). Integrated regulation of the many processes involved in the dynamics of the thylakoid membrane is not yet fully understood (Tikkanen and Aro, 2014). It has been shown that the PSII-LHCII supercomplex phosphorylation level affects the distance between the grana stacks as well as the size and suggested that complexes in opposite orientation repel each other, causing unstacking of the grana (Fristedt *et al.*, 2009). Thus, the reduced levels of PSII (D1 and D2) observed in *psb33* mutant plants exposed to blue light (Figure 6), could explain changes to the thylakoid structure of *psb33*, seen by transmission electron microscopy (Figure 4). The distinctly swollen stroma lamella might negatively influence the rate of movement of proteins and compounds, restrict PSII quality control, and enhance damage to the complex (Kirchhoff, 2014). The altered PSII superstructure and PSII level in *psb33* might have further enhanced the growth phenotype.

UVA light reduces Photosystem II proteins in psb33.

Two previous observations have been linked to blue- and UVA light that can explain *psb33* dwarf phenotype. First, the Mn_4CaO_5 can directly absorb UV light and initiate the photoinactivation process (Ohnishi *et al.*, 2005). It could be possible, but not likely, that PSB33 protects the Mn cluster, due to restricted access by the OEC proteins. However, our data on intra PSII electron flow do not support this hypothesis (Figure 7). Second, blue- and UVA light-mediated signals have been linked to increased D2 and CP43 gene expression in mature chloroplasts (Christopher and Mullet, 1994) and this is consistent with our results: we observed an up-regulation of D1 and D2 PSII proteins in wild type plants exposed to blue/UVA light (Figure 6A). In contrast, D1 and D2 were down-regulated in *psb33* mutant plants in blue/UVA.

PSB33 location is largely in non-appressed thylakoid regions, and with its head domain exposed to the stroma (Fristedt *et al.*, 2015), and is part of a larger protein complex (Fristedt *et al.*, 2017). PSB33 is likely part of a signaling mechanism involved in the rejuvenation of PSII. A hypothetical scenario would be that this mechanism is either triggered by UVA light or develops as a consequence of plant exposure to blue and UV light, possibly as a result of re-adjusted redox regulation. Such a signaling cascade may eventually induce a

conformational change on PSB33, which triggers downstream signaling for transcription of various PSII proteins.

Conclusions

The accumulated data show that PSB33 has an important role in sustaining a functional PSII pool in blue light, especially in UVA light. Although the severe growth phenotype of *psb33* plants in blue light is connected to misregulated phosphorylation and migration of Lhcb proteins, this appears not to be a causal relationship. The exact molecular mechanism of how PSB33 protein senses UV-light and initiates the photosynthetic response remains to be investigated.

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Data Availability Statement

The data supporting the findings of this study are available from the corresponding author, Björn Lundin Burmeister, upon request.

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Author contribution

AN and BL designed the experiments. AN, DB, and BL performed light experiments and growth of plants. AN and BL performed pigment, chlorophyll *a* fluorescence, thylakoid membrane preparation, SDS-PAGE, immunoblotting, and ROS determination. AP and ON performed hormonal analysis. OJ performed transmission electron microscopy. FM performed thermoluminescence and low-temperature electron paramagnetic resonance spectroscopy. All authors contributed to the analysis of results and writing of the manuscript.

Conflict of Interest

Björn Burmeister is employed at AstraZeneca, Respiratory & Immunology at the time of publication and declares that there is no conflict of interest that has effected this publication.

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Tables

Table 1. Pigment composition in plants grown in different lights.

	Total chlorophyll (mg g ⁻¹)		Carotenoids (mg g ⁻¹)		Chl a/b	
	wt	<i>psb33</i>	wt	<i>psb33</i>	wt	<i>psb33</i>
Fluorescent white	1.11±0.03	0.92±0.07**	0.23±0.01	0.18±0.02**	3.97±0.07	3.85±0.03**
Blue	0.87±0.07	0.74±0.08*	0.18±0.01	0.16±0.02	4.71±0.07	4.33±0.42
Red	0.96±0.23	0.96±0.09	0.20±0.05	0.21±0.02	4.61±0.09	4.63±0.20
Blue and red	1.07±0.09	1.07±0.09	0.22±0.02	0.23±0.02	4.57±0.05	4.46±0.13

Table 1. Pigment composition was determined in leaf tissue from five-week-old plants. Plants were grown for two weeks in white fluorescent light and then transferred to LED chambers emitting light of different wavelengths of either blue [400,420,440] nm or red [630,650] nm light, or a combination of the two and grown for additional three weeks. Control plants were kept in white fluorescent light throughout the growth period. Asterisks indicate statistically significantly different to wild type under the specified growth condition (* $p < 0.05$, ** $p < 0.01$ Student's t -test, $n=6$).

Figure legends

Figure 1. The growth of the *psb33* is strongly inhibited by blue monochromatic light. Plants were grown for five weeks in white fluorescent light at $120 \mu\text{mol m}^{-2}\text{s}^{-1}$, or alternatively, grown for two weeks in white fluorescent light and then transferred to blue or red monochromatic light, or a combination of the two (left panel), and grown for additional three weeks before photos were taken (mid panel) and fresh weight (FW) determined (right panel). Percentage values in the right panel indicate relative size to wild type (wt). Means and standard deviation are shown ($n=11-24$). Letters indicate a statistically significant difference between groups (one-way ANOVA, Bonferroni-Holm posthoc test, $p<0.05$).

Figure 2. *Fv/Fm* in plants under blue and red monochromatic light. **A.** Plants were grown for five weeks in fluorescent white light at $120 \mu\text{mol m}^{-2}\text{s}^{-1}$, or alternatively, grown for two weeks in fluorescent white light and then transferred to LED growth chambers emitting light in different wavelengths of either blue [400,420,440] nm or red [630,650] nm light, or a combination of the two, and grown for additional three weeks before *Fv/Fm* was determined ($n=14-20$). **B and C.** Plants were grown for four weeks in fluorescent white light and then transferred LED growth chambers emitting light in different wavelengths of either blue [400,420,440] nm (**B**) or red [630,660] nm light (**C**) and kept for three days, then returned to fluorescent white light for three days. *Fv/Fm* values were determined at regular intervals ($n=12$). **D and E** Plants were grown for four weeks in fluorescent white light and then transferred LED growth chambers emitting light in different wavelengths of either blue [400/420/440] nm (**D**) or red [630/660] nm light (**E**) and *Fv/Fm* was measured before (0), 3 and 7 days after transfer ($n=12-15$). N.D. not determined. Means and standard deviation are shown.

Figure 3. Changes in phytohormone levels are observed in *psb33* grown in blue or red monochromatic light. Hormones levels were determined in leaf tissue (fresh weight, FW) from plants grown in fluorescent white light (FL) or under LED emitting light in different wavelengths of blue [400,420,440] nm or red [630,660] nm light. Means and standard deviation are shown ($n=4$). Asterisks indicate a statistically significant difference in the

mutant line (*psb33*) versus the wild type (wt) as determined by one-way ANOVA with Bonferroni-Holm posthoc test ($p < 0.01$). **A** indole acetic acid (IAA); **B** oxindole-3-IAA (oxIAA); **C** asparagine conjugated IAA (IAA-Asp); **D** glutamate conjugated IAA (IAA-Glu); **E** abscisic acid (ABA); **F** salicylic acid (SA); **G** jasmonic acid (JA); **H** isoleucine conjugate JA (JA-Ile); **I** and 12-oxo-phytodienoic acid (*cis*-OPDA).

Figure 4. Thylakoid ultrastructure of *psb33* and wild type plants grown in different lights.

Representative transmission electron micrographs are shown for leaf chloroplasts from five-week-old wild type (wt), and *psb33* mutant plants fixed two hours after the onset of illumination. The thylakoids of the wild type and *psb33* mutant are similar when grown in fluorescent white light or under LED emitting red light in the wavelength of [630,660] nm. When grown under LED emitting blue light [400,420,450] nm), *psb33* displays swollen thylakoid, rarely observed in the wild type. Scale bars, 2 μm (**A**) and 500 nm (**B**).

Figure 5. *psb33* plants are sensitive to UVA light. Plants were grown for two weeks in fluorescent white light and then transferred to LED emitting blue light in different wavelengths [400] or [400,420] or [400,420,440] nm) but with similar intensity (120 PFD) where they were grown for three more weeks before photos were taken (**A**), fresh weight determined (**B**), and *Fv/Fm* measured (**C**). Means and standard deviation are shown where $n=13-15$ and $n=18-21$, in **B** and **C** respectively. Asterisks indicate statistically significantly different to wild type under the specified growth condition or between samples marked by brackets (one-way ANOVA, Bonferroni-Holm posthoc test, $p < 0.01$). Data used for statistical analysis of fresh weight were transformed (square root) to meet equal variance.

Figure 6. Western blots of total protein extracts from plants grown in different light conditions.

Whole leaf protein extracts were prepared from plants grown for three weeks in either fluorescent white light or under LED emitting light in different wavelengths of (**A**) blue light [400,420,440] or [420,440] nm), red light [630/650] nm and blue and red light supplemented with far-red light [400,420,440,730] nm or [630,650,730] nm and (**B**) blue light [400], [420,440] or [400,420,440] nm). Proteins were separated with SDS-PAGE, and loading was based on chlorophyll content.

Figure 7. Analysis of electron transport components in photosystem II from thylakoid membranes from wt (black traces/spectra) and *psb33* mutant (red traces/spectra). **A** Flash-induced fluorescence decay kinetic in the absence (left panel) or the presence of 20 μ M DCMU (right panel). **B** Thermoluminescence glow curves in the absence (left panel) or the presence of 40 μ M DCMU (right panel). EPR measurements of the **C**, Tyrosine D radical, **D**, the S_2 state of the water oxidizing complex and **E**, the oxidized form of Cytochrome b_{559} in the g_z region before (dotted line) and after illumination at 77 K (solid line). EPR conditions: microwave frequency: 9.35 GHz, microwave power 1.3 μ W and temperature 15 K for **C**, 10 mW and 7 K for **D**, and 5 mW and 15 K for **E**.

Figure 8. ROS staining of plants grown in different wavelengths of blue light. 7-days-old seedlings were transferred from fluorescent white light to LED plant growth chambers emitting light in different wavelengths of blue light [400] or [420,450] nm and grown for 2 weeks before staining against H_2O_2 (3,3'-Diaminobenzidine, DAB) and superoxide radicals (nitroblue tetrazolium, NTB). The photo show representative plants from 5-8 stained plants.

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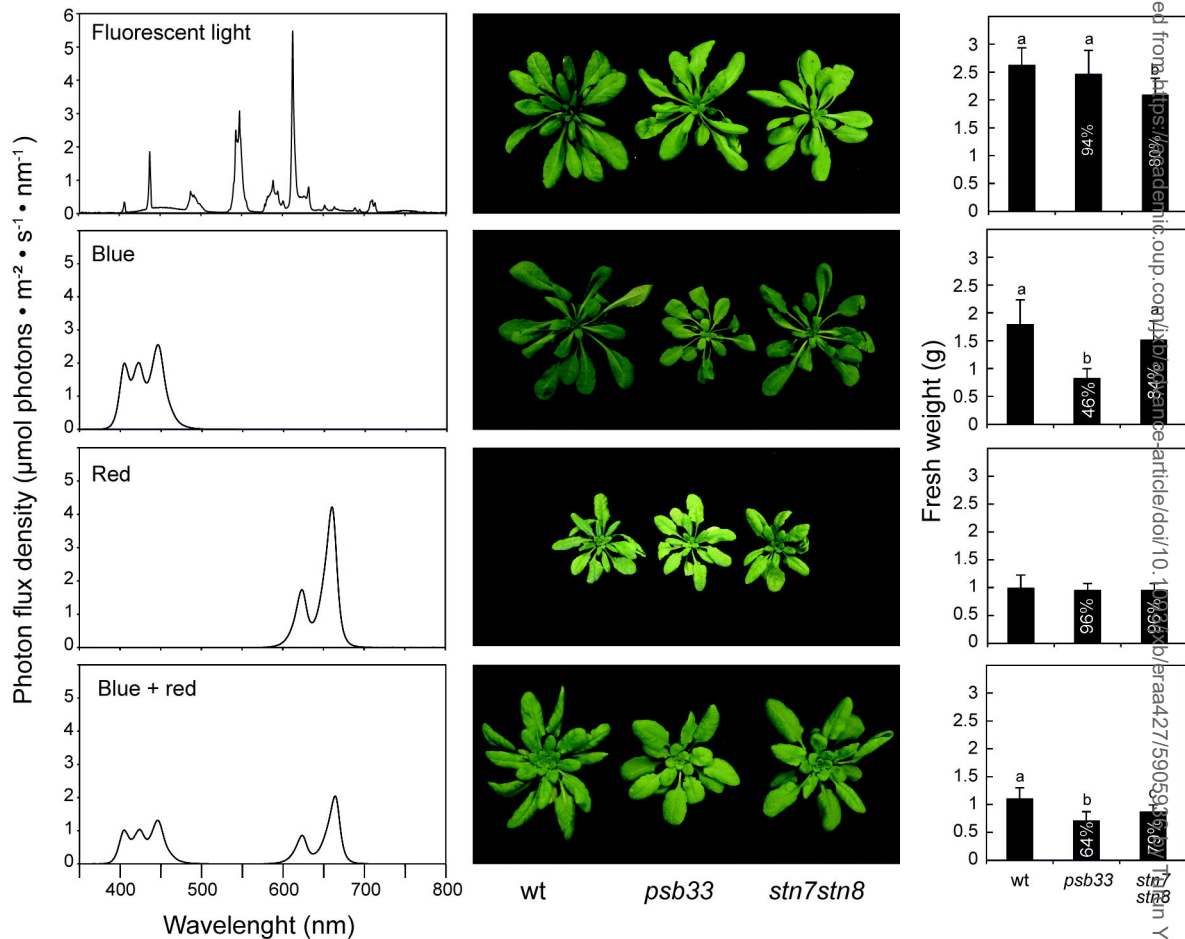


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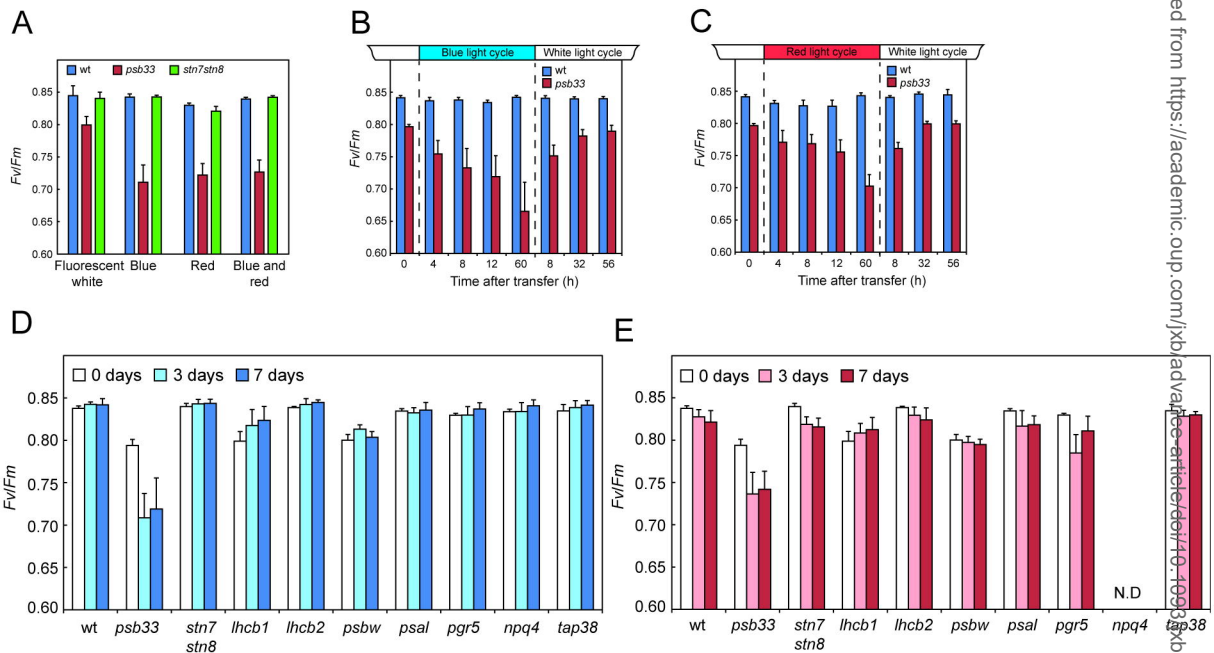


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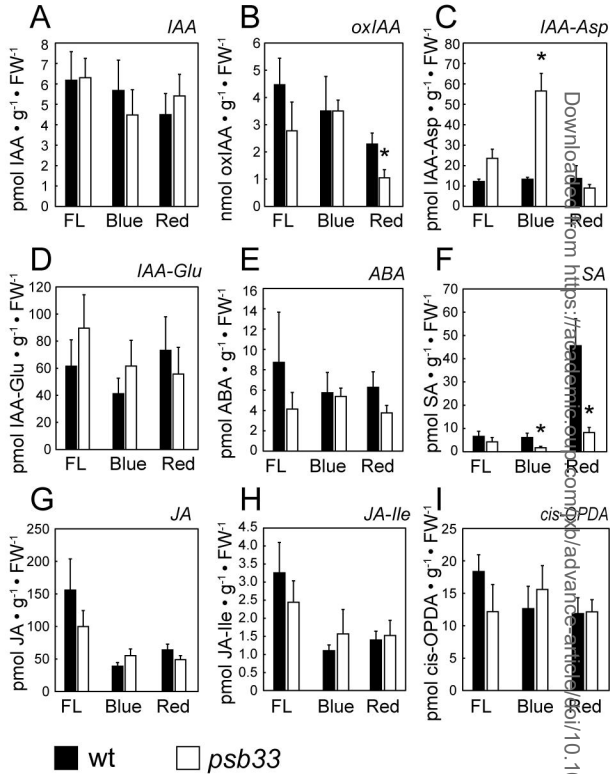


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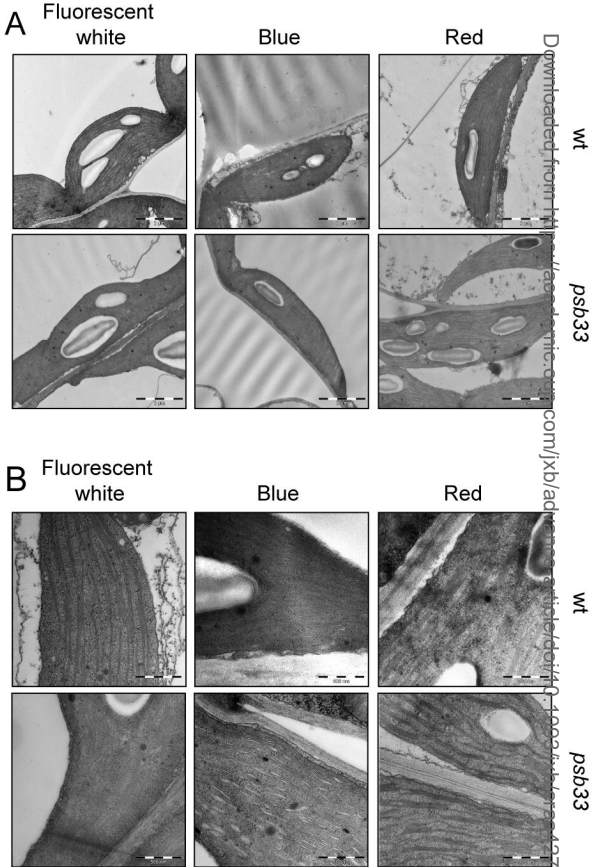
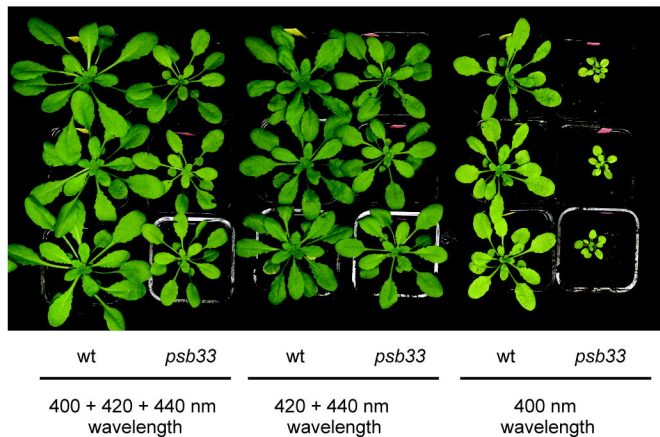


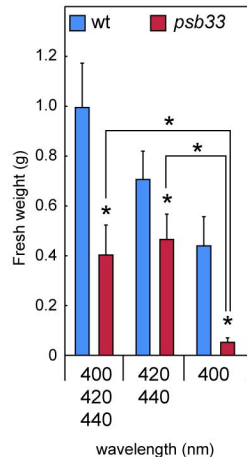
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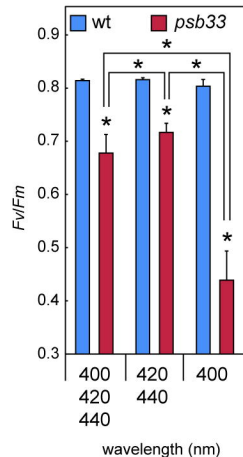


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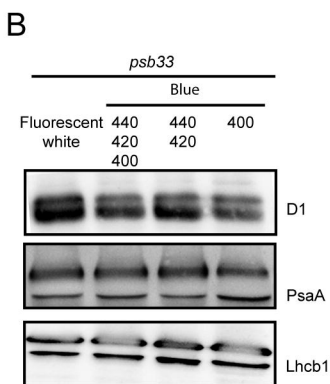
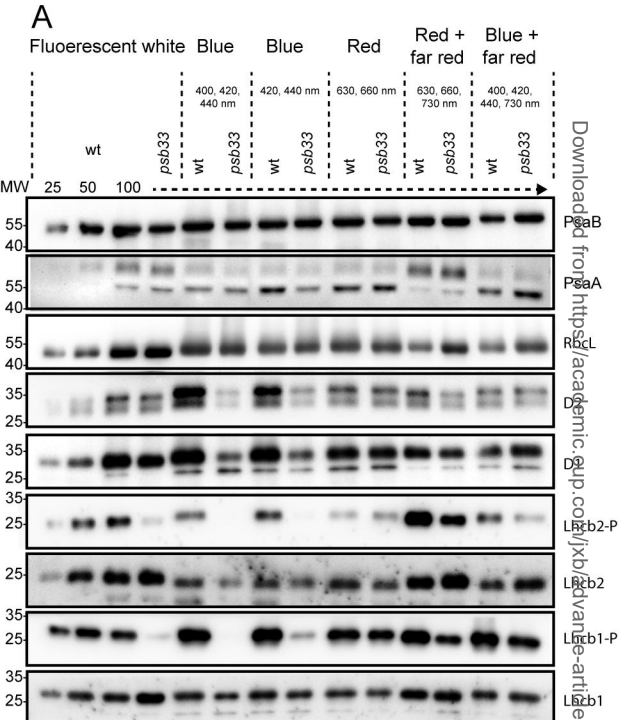


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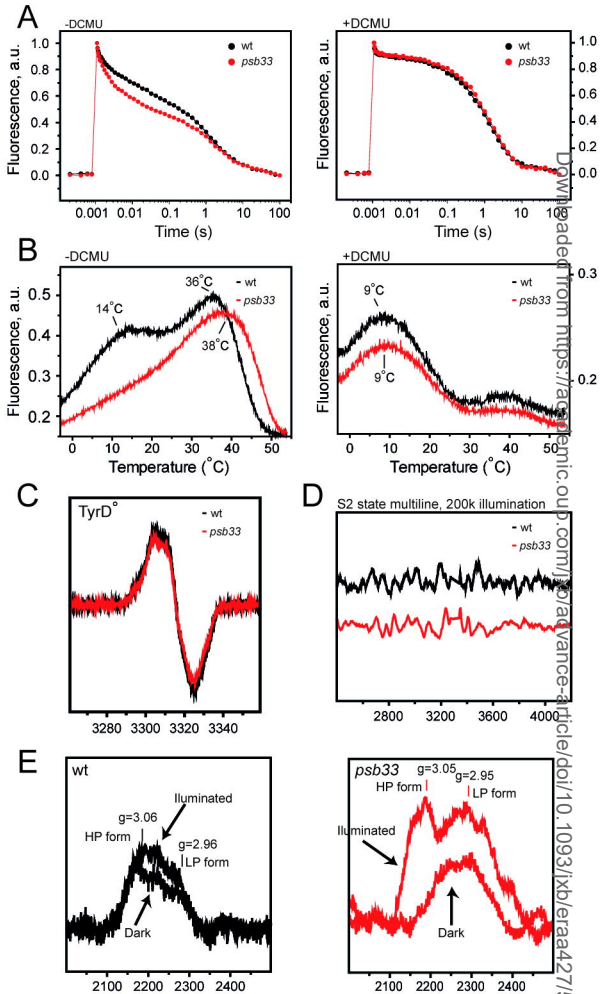


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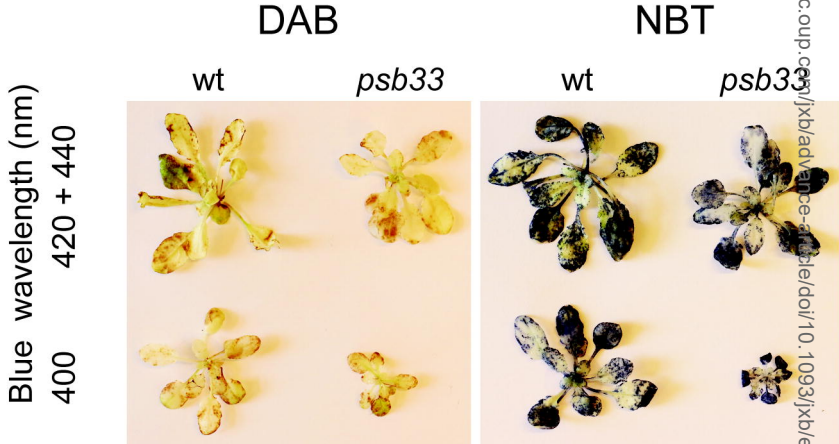


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