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Essential Role of Acyl-ACP Synthetase in Acclimation of the Cyanobacterium *Synechococcus elongatus* Strain PCC 7942 to High-Light Conditions

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Most organisms capable of oxygenic photosynthesis have an *aas* gene encoding an acyl-acyl carrier protein synthetase (Aas), which activates free fatty acids (FFAs) via esterification to acyl carrier protein. Cyanobacterial *aas* mutants are often used for studies aimed at photosynthetic production of biofuels because the mutation leads to intracellular accumulation of FFAs and their secretion into the external medium, but the physiological significance of the production of FFAs and their recycling involving Aas has remained unclear. Using an *aas*-deficient mutant of *Synechococcus elongatus* strain PCC 7942, we show here that remodeling of membrane lipids is activated by high-intensity light and that the recycling of FFAs is essential for acclimation to high-light conditions. Unlike wild-type cells, the mutant cells could not increase their growth rate as the light intensity was increased from 50 to 400 μmol photons m^{-2} s^{-1}, and the high-light-grown mutant cells accumulated FFAs and the lysolipids derived from all the four major classes of membrane lipids, revealing high-light-induced lipid deacylation. The high-light-grown mutant cells showed much lower PSII activity and Chl contents as compared with the wild-type cells or low-light-grown mutant cells. The loss of Aas accelerated photodamage of PSII but did not affect the repair process of PSII, indicating that PSII is destabilized in the mutant. Thus, Aas is essential for acclimation of the cyanobacterium to high-light conditions. The relevance of the present findings to biofuel production using cyanobacteria is discussed.

Keywords: Acyl-ACP synthetase • Biofuel production • Cyanobacteria • High-light acclimation • Photoinhibition of PSII.

Abbreviations: Aas, acyl-acyl carrier protein synthetase; ACP, acyl carrier protein; BN-PAGE, blue native PAGE; DGDG, digalactosyldiacylglycerol; DGMG, digalactosylmonoacylglycerol; HL, high light; FFA, free fatty acid; LC, liquid chromatography; LL, low light; MGDG, monogalactosyldiacylglycerol; MGMG, monogalactosylmonoacylglycerol; MS, mass spectrometry; pBQ, 1,4-benzoquinone; PG, phosphatidylglycerol; SQDG, sulfoquinovosyldiacylglycerol; SQMG, sulfoquinovosylmonoacylglycerol; WT, wild type.

Introduction

The *aas* gene encodes acyl-acyl carrier protein synthetase (Aas), which activates free fatty acids (FFAs) via esterification to acyl carrier protein (ACP). Using the recombinant protein and the knockout mutant strains defective in the gene, the biochemical function of Aas and its involvement in recycling of FFAs have been established in the cyanobacteria *Synechocystis* sp. strain PCC 6803 and *Synechococcus elongatus* strain PCC 7942 (Kaczmarzyk and Fulda 2010). It has also been shown that phosphatidylglycerol (PG) and sulfoquinovosyldiacylglycerol (SQDG) can serve as the source of FFAs in vivo, although the lipase(s) involved in the deacylation reaction is yet to be identified. Because of the deficiency in FFA recycling, the Aas-null mutants accumulate FFAs intracellulary and secrete them into the external medium. Cyanobacterial *aas* mutants have therefore been used for studies aimed at photosynthetic production of biofuels (Liu et al. 2011, Ruffing and Jones 2012, Ruffing 2014). However, the physiological significance of the role of Aas in cyanobacteria remains unclear, because no noticeable growth phenotype has been found for the *aas*-deficient mutants (Kaczmarzyk and Fulda 2010). The physiological significance of the FFA formation is also unknown. To exploit the ability of the Aas-deficient mutants to produce FFAs for biofuel production, however, it is essential to clarify the physiological relevance of the formation and recycling of FFAs in cyanobacterial cells. In this study, we show that the growth phenotype of an *aas*-deficient mutant of *S. elongatus* strain PCC 7942 becomes manifest under high-intensity light. Under high-light (HL) conditions, the mutant cells are shown to accumulate FFAs and the lysolipids derived from all four major lipid classes of cyanobacteria, revealing HL-induced remodeling of the membrane lipids. It is shown that the mutant is hypersensitive to photoinhibition because of reduced stability of PSII. Aas is thus

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shown to be essential for acclimation of the cyanobacterial cells to HL conditions. The relevance of the findings to the development of the FFA production system using cyanobacteria is discussed.

Results

Effects of Aas deficiency on growth of *S. elongatus* strain PCC 7942

To investigate the physiological role of Aas in *S. elongatus* PCC 7942, we generated an Aas-deficient mutant (hereafter referred to as dAS1) by deleting an internal 667 bp from the *aas* coding region (**Supplementary Fig. S1**) and compared its growth characteristics with those of the wild type (WT) (**Fig. 1**). In all the experiments described herein, liquid cultures were aerated with CO_2-enriched air (2%, v/v) so that growth would not be limited by CO_2 supply. Under low-light (LL) conditions (50 μmol photons m^{-2} s^{-1}), growth of dAS1 was slightly slower than that of the WT (**Fig. 1Aa**) but the appearance of the mutant cultures was similar to that of the WT cultures at $t = 48$ h (**Fig. 1Ba**). While the specific growth rate of the WT was increased as the light intensity was increased from 50 to 400 μmol m^{-2} s^{-1}, that of dAS1 reached a maximum at around 200 μmol m^{-2} s^{-1} and then declined to a level similar to that observed under the LL conditions (**Fig. 1C**). As a result, the slow growth phenotype of the mutant was most prominent under the HL conditions (400 μmol photons m^{-2} s^{-1}).

High light induced accumulation of FFAs in dAS1 cells

The *aas*-deficient mutants of cyanobacteria accumulate FFAs and secrete them into the culture medium (Kaczmarzyk and Fulda 2010). In the WT cells, the total amount of intracellular FFA in the linear phase of growth ($t = 48$ h) was about 500 ng per 10^8 cells, with palmitic acid (16:0) and stearic acid (18:0) being the major constituents, and was not affected by the light conditions (**Fig. 2A, B**). The total FFA content in the dAS1 mutant grown under LL conditions was somewhat higher than that in the WT, and was increased by 2.3-fold by growth under HL conditions (**Fig. 2A**). The contents of 16:0 and palmitoleic acid (16:1) were much higher in the HL-grown dAS1 cells than in the LL-grown cells (**Fig. 2B**), while the C18 fatty acids did not show HL-induced accumulation. As a result, 16:0 comprised a much larger portion of FFAs in the HL-grown dAS1 cells (59% of the total FFAs) than in WT and LL-grown dAS1 cells (29–36% of the total FFAs).

Accumulation of large amounts of FFA in the medium of *aas*-deficient mutant cultures takes place in the later phases of growth ($t > 200$ h), attaining a concentration as high as 200 μmol l^{-1} (Ruffing and Jones 2012). At $t = 48$ h, the extracellular FFA concentrations in the LL-grown WT and dAS1 cultures were low, being 3 and 4 μmol l^{-1}, respectively. The extracellular FFA concentration of WT cultures was not affected by the increase in light intensity up to 400 μmol m^{-2} s^{-1}, but that of the mutant cultures was increased to about 7 μmol l^{-1} (**Supplementary Fig. S2**). The higher level of extracellular FFAs in the HL-grown dAS1 cells than in the LL-grown cells seemed to reflect the increased accumulation of intracellular FFA in the former cells.

High light induced accumulation of lysolipids

Fig. 2C shows the effects of the HL conditions on the contents of lysolipids and diacyl lipids in the WT and dAS1. In the WT, the content of sulfoquinovosylmonoacylglycerol (SQMG) was three times higher in HL-grown cells than in LL-grown cells, but the contents of lysoPG, monogalactosylmonoacylglycerol (MGMG) and digalactosylmonoacylglycerol (DGMG) were not affected by the light conditions. The LL-grown dAS1 cells contained a 2.5-fold larger amount of lysoPG as compared with the WT, while the contents of the other lysolipids were similar to those in LL-grown WT cells, suggesting that the higher content of FFAs in the LL-grown dAS1 mutant was due to deacylation of PG. Under the HL conditions, the dAS1 mutant accumulated larger amounts of all four lysolipids than under

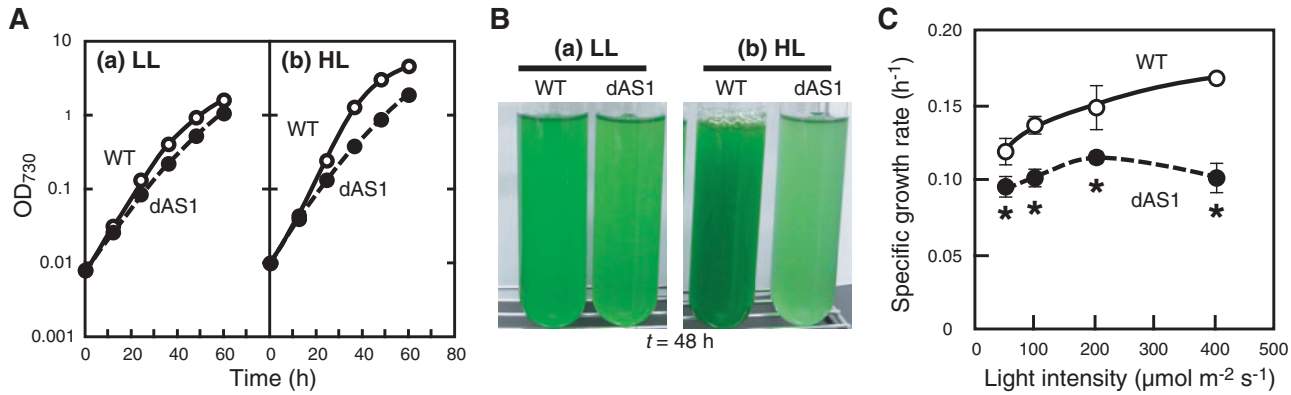

Fig. 1 Effects of *aas* deficiency on growth of *S. elongatus* PCC 7942. (A) Growth curves of the WT (open circles) and dAS1 (filled circles) under low-light (a, LL; 50 μmol m^{-2} s^{-1}) and high-light (b, HL; 400 μmol m^{-2} s^{-1}) conditions. OD_{730}, optical density at 730 nm. (B) Appearance of the cultures in (A) at $t = 48$ h. (C) Effects of light intensity on the specific growth rate of the WT (open circles) and dAS1 (filled circles). Data shown are the means ± SD from 3–5 biological replicates ($n = 3$–5). Asterisks indicate significant differences compared with the WT under the same light intensity ($P < 0.05$, Student's *t*-test).

the LL conditions. The HL-induced increase of FFA and lysolipids revealed that deacylation of membrane lipids is stimulated under the HL conditions.

As previously reported (Ruffing and Jones 2012), the contents of digalactosyldiacylglycerol (DGDG) and SQDG, but not those of PG and monogalactosyldiacylglycerol (MGDG), were lower in the Aas-deficient mutant than in the WT (**Fig. 2Cg, h**), but their contents were not significantly affected by HL illumination. Unlike the other lipids, PG was found to be decreased under the HL conditions regardless of the presence or absence of Aas (**Fig. 2Ce**).

Fig. 3 shows the compositions of lipid molecular species in the four major lipid classes in WT and dAS1 cells grown under the LL and HL conditions for 48 h. The predominance of the C32 molecular species (32:0, 32:1 and 32:2) carrying two C_{16} fatty acyl chains was consistent with the previously reported fatty acid compositions of membrane lipids in *S. elongatus* (Murata et al. 1992, Kaczmarzyk and Fulda 2010, Ruffing and Jones 2012). In the WT, the light conditions did not significantly affect the composition of lipid molecular species in MGDG, DGDG and SQDG, but for PG, growth under HL significantly decreased the content of the C34 molecular species (34:1) carrying one each of C_{16} and C_{18} fatty acyl chains. The profiles of lipid molecular species in LL-grown dAS1 were similar to the corresponding profiles in the WT. Growth of dAS1 under the HL conditions caused a significant increase in the C30 molecular species (30:0 and 30:1) carrying one each of C_{14} and C_{16} fatty acyl chains with concomitant decreases in the C32 and C34 molecular species, indicating decreases in the average chain length of the fatty acyl moieties of membrane lipids. The content of C_{18} fatty acid in HL-grown dAS1 was almost negligible, which accounted for the predominance of the C_{16} fatty acids in the intracellular FFA fraction of HL-grown dAS1 cells (**Fig. 2B**).

Effects of Aas deficiency on photosynthetic activity

Declines of the Chl content and the photosynthetic yield have been reported to become manifest in the stationary phase of growth of an *aas* mutant of *S. elongatus* strain PCC 7942 (Ruffing and Jones 2012). Even during the seemingly normal growth of dAS1 under the LL conditions ($t = 48$, see **Fig. 1A, B**), however, the cellular Chl content was slightly lower than

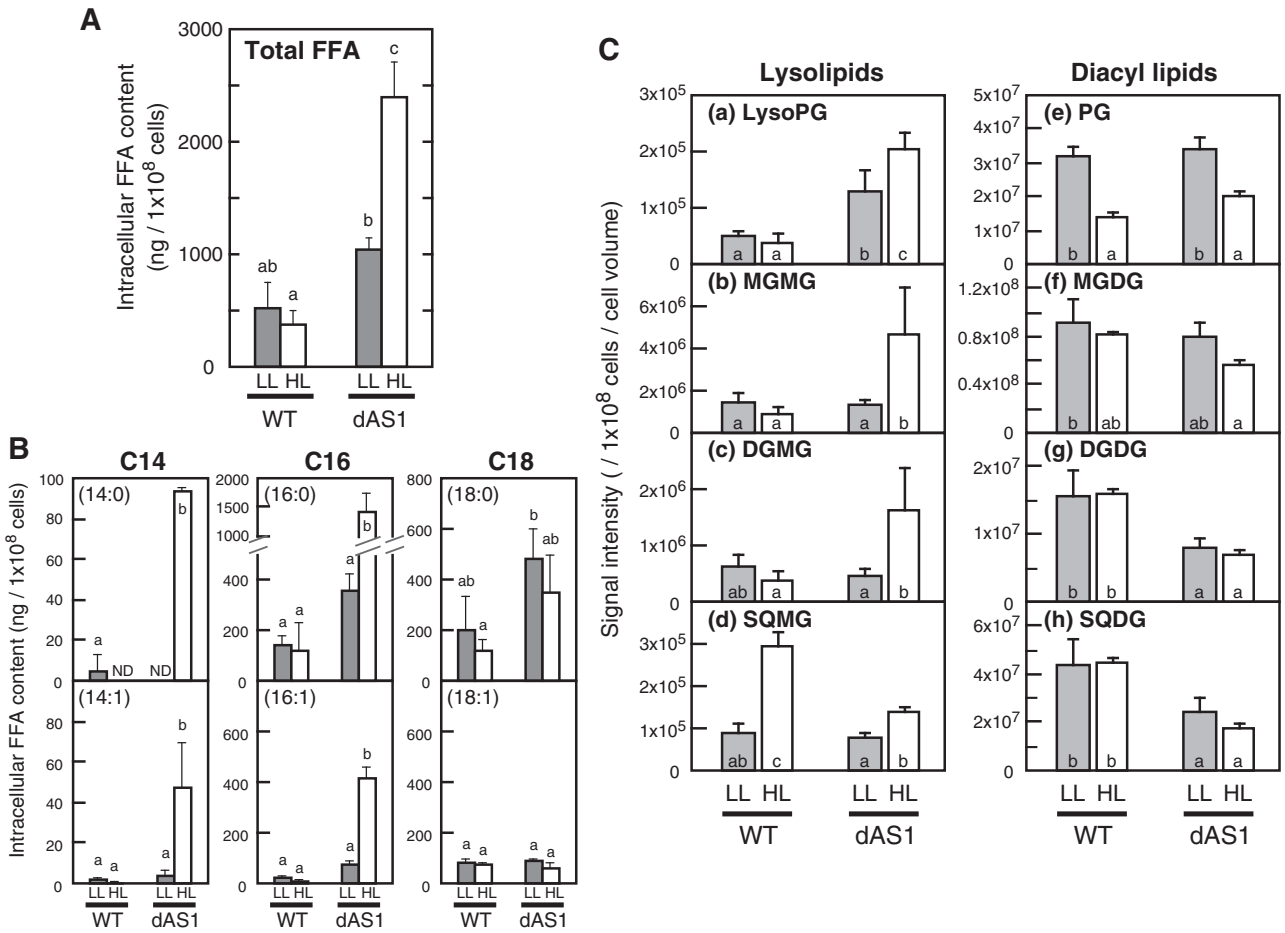

Fig. 2 Effects of light intensity on the contents of FFAs, lysolipids and diacyl lipids in the cells of the WT and dAS1. The cells were grown under LL (50 μmol m^{-2} s^{-1}) or HL (400 μmol m^{-2} s^{-1}) conditions for 48 h. (A) Total FFA content in the cells. (B) Contents of C14, C16 and C18 FFAs in the cells. (C) The contents of lysolipids and diacyl lipids in the cells. (a) LysoPG, (b) MGMG, (c) DGMG, (d) SQMG, (e) PG, (f) MGDG, (g) DGDG, (h) SQDG. Data shown are the means ± SD from biological triplicates ($n = 3$). Different letters denote significant differences ($P < 0.05$, Tukey's test).

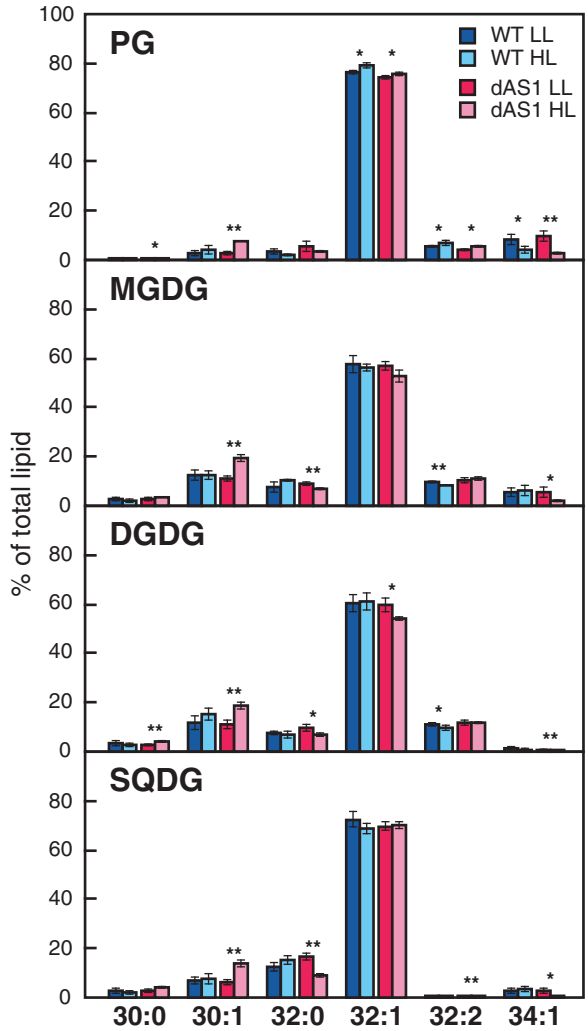

Fig. 3 Composition of lipid molecular species in the four major lipid classes of the cells of the WT and dAS1. The cells were grown under LL (50 μmol m^{-2} s^{-1}) or HL (400 μmol m^{-2} s^{-1}) conditions for 48 h. Data shown are the means ± SD from biological triplicates ($n = 3$). Asterisks indicate significant differences between the results from the different light conditions (*$P < 0.05$, **$P < 0.01$, Student's *t*-test).

that of the WT (**Fig. 4A**). The O_2-evolving activity of PSII, as measured with 1,4-benzoquinone (pBQ) as the electron acceptor, was also lower in dAS1 than in the WT by about 30% (**Fig. 4Ba**). Under the HL conditions, the Chl content of the mutant, but not that of the WT, was markedly decreased (43% of the level under LL conditions). The PSII O_2-evolving activity was decreased under the HL conditions in both the WT and dAS1, but the extent of decrease was larger in dAS1 than in the WT. In contrast, O_2 evolution measured with CO_2 as the electron acceptor, which represents the overall rate of CO_2 fixation, was not significantly affected by HL irradiation in the *aas*-deficient mutant (**Fig. 4Bb**). The photosynthetic yield of PSII, as determined by measuring the F_v/F_m ratio, decreased much more sharply in the mutant than in the WT as the light intensity during growth was increased (**Fig. 4C**). The enhanced photoinhibition of PSII in dAS1 indicated that Aas is required for normal acclimation of *S. elongatus* to HL conditions.

Increased sensitivity of PSII to photodamage in dAS1

The extent of photoinhibition is determined by the balance of photodamage of PSII and repair of damaged PSII (Nishiyama and Murata 2014). To determine which of these two processes was affected by the *aas* deficiency, we examined the time courses of photoinhibition in the presence and absence of chloramphenicol, which inhibits the repair process of damaged PSII (**Fig. 5**). In the presence of chloramphenicol, PSII activity was reduced to near-zero levels in both the WT and dAS1, but the HL-induced decline of O_2 evolution activity was much faster in the dAS1 mutant than in the WT, revealing enhanced photodamage to PSII in the dAS1 mutant (**Fig. 5A**). Faster decline of PSII activity in dAS1 than in the WT was also observed in the absence of chloramphenicol, but the extent of photoinhibition after 60 min of HL illumination was similar to that in the WT (**Fig. 5B**). Importantly, the rate of recovery of the O_2 evolution activity during the subsequent LL period was similar between the WT and the dAS1 mutant, suggesting that the repair process was not affected by the *aas* deficiency. These results suggested that the *aas* deficiency caused destabilization of PSII under the HL conditions.

Effects of high light and Aas deficiency on oligomerization state of photosystems

To gain further insight into the effects of *aas* deficiency on photosynthesis, the oligomerization state of the PSI and PSII complexes was analyzed by means of blue native (BN)-PAGE (**Fig. 6A**). The thylakoid membranes isolated from LL-grown WT cells showed clear bands of PSI trimers, PSII dimers and mixture of PSI and PSII monomers as previously reported (Watanabe et al. 2009). Similar oligomerization states of the photosystems were observed in the LL-grown dAS1 mutant. While the electrophoretic profile of the photosystems in the WT was not affected by growth under the HL conditions, that of the HL-grown dAS1 mutant showed a smeary pattern with poor resolution of the green bands. It was nevertheless clear that the level of the uppermost band corresponding to the PSI trimer was much lower than that in the LL-grown cells. Western blot analysis showed that the levels of the PsbA and PsbB proteins comprising the PSII reaction center complex were not affected by the *aas* deficiency and/or illumination under HL, while the levels of the PsaA protein comprising the PSI reaction center complex were significantly decreased in the dAS1 mutant irrespective of the light conditions (**Fig. 6B**). Low-temperature (77 K) Chl fluorescence spectra of the isolated thylakoid membranes provided further support for the abnormal state of photosystems in the dAS1 mutant grown under the HL conditions (**Fig. 6C**). The emission peak arising from PSII showed a significant increase and a blue shift from 699 to 694 nm, and the peak at 715 nm arising from PSI showed a decrease. These results suggested that HL illumination not only reduced the PSII activity of dAS1 but also changed the organization of the whole photosynthetic apparatus. Similar changes in the states of photosystems, as revealed by decreased levels of PSI trimer and an increased PSII fluorescence level relative to that

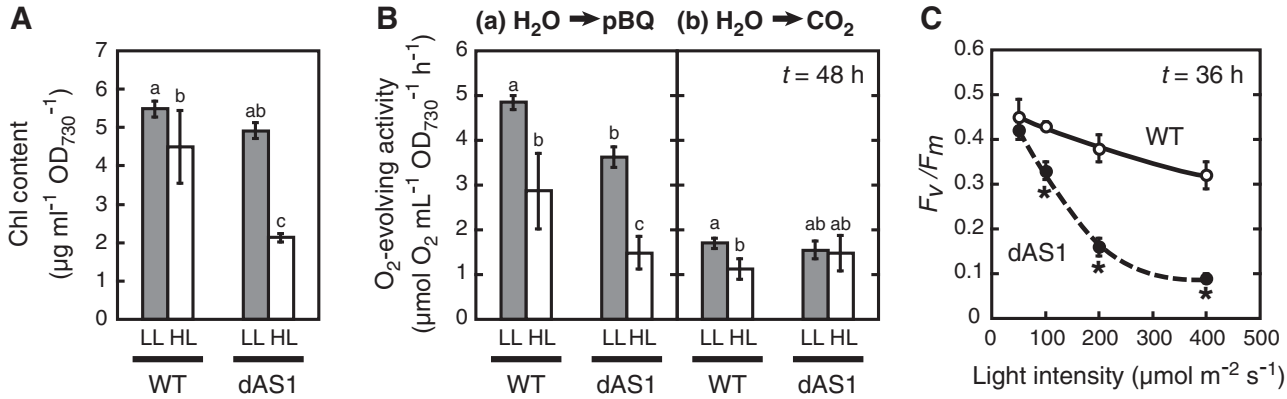

Fig. 4 Effects of light intensity on the Chl content (A), photosynthetic O_2-evolving activity (B) and photosynthetic yield (C) of the WT and dAS1. (A and B) Cells were grown under LL (50 μmol m^{-2} s^{-1}) or HL (400 μmol m^{-2} s^{-1}) conditions for 48 h. Photosynthetic O_2-evolving activity was measured with 1 mM pBQ (a) or CO_2 (b) as the electron acceptor under illumination at 1,000 μmol m^{-2} s^{-1} for 2 min. (C) WT (open circles) and dAS1 (filled circles) cells were grown under the indicated light intensities for 36 h. Data shown are the means ± SD from five (A and B) and three (C) biological replicates ($n = 5$ and 3). Different letters denote significant differences from a Tukey's test ($P < 0.05$). Asterisks indicate significant differences compared with the WT under the same light intensity ($P < 0.05$, Student's *t*-test).

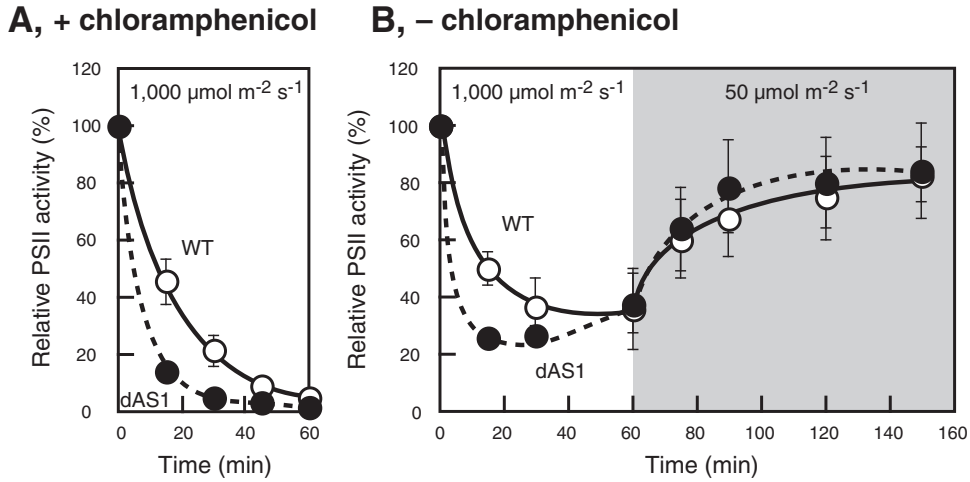

Fig. 5 Effects of *aas* deficiency on the sensitivity of PSII to photoinhibition. WT (open circles) and dAS1 (filled circles) grown under LL (50 μmol m^{-2} s^{-1}) conditions for 48 h were incubated under 1,000 μmol m^{-2} s^{-1} light conditions for 60 min in the presence (A) or absence (B) of 30 μg ml^{-1} chloramphenicol. In the experiment shown in (B), recovery of photosynthetic activity was monitored under the LL conditions. Photosynthetic O_2-evolving activity was measured with 1 mM pBQ as the electron acceptor. One hundred percent activity for the WT and dAS1 was in the range shown in **Fig. 4B**. Data shown are the means ± SD from biological triplicates ($n = 3$).

of PSI fluorescence, have been reported in Mn-depleted cells suffering from photoinhibition (Salomon and Keren 2011). The changes observed in dAS1 seemed to have resulted from prolonged incubation of the cells under photoinhibited conditions because the severly photoinhibited dAS1 cells after short-term exposure to high light (**Fig. 5**) retained normal oligomerization states of the photosystems (**Supplementary Fig. S3**).

Discussion

FFA production using genetically engineered cyanobacteria is thought to be a promising method of photosynthetic production of biofuels; Aas-deficient mutants with or without introduced thioesterase gene(s) have been shown to excrete FFAs to the external medium in the stationary phase of growth (Liu et al. 2011, Ruffing and Jones 2012, Ruffing 2014). However, FFAs act as surfactants and have adverse effects on biological membranes, e.g. disruption of the electron transport chain, interference with oxidative phosphorylation, and inhibition of enzymes and uptake of nutrients (Desbois and Smith 2010). It is therefore not surprising that the engineered cyanobacterial strains suffer from the toxicity of FFAs after reaching the stationary phase of growth (Ruffing 2014). On the other hand, it is rather surprising that no noticeable growth defect has been detected during growth of the cells in the logarithmic or linear phase of growth (Kaczmarzyk and Fulda 2010, Ruffing and Jones 2012). It should be noted that the previous studies were carried out under LL conditions (30–38 μmol photons m^{-2} s^{-1}). The present results show that the *aas*-deficient

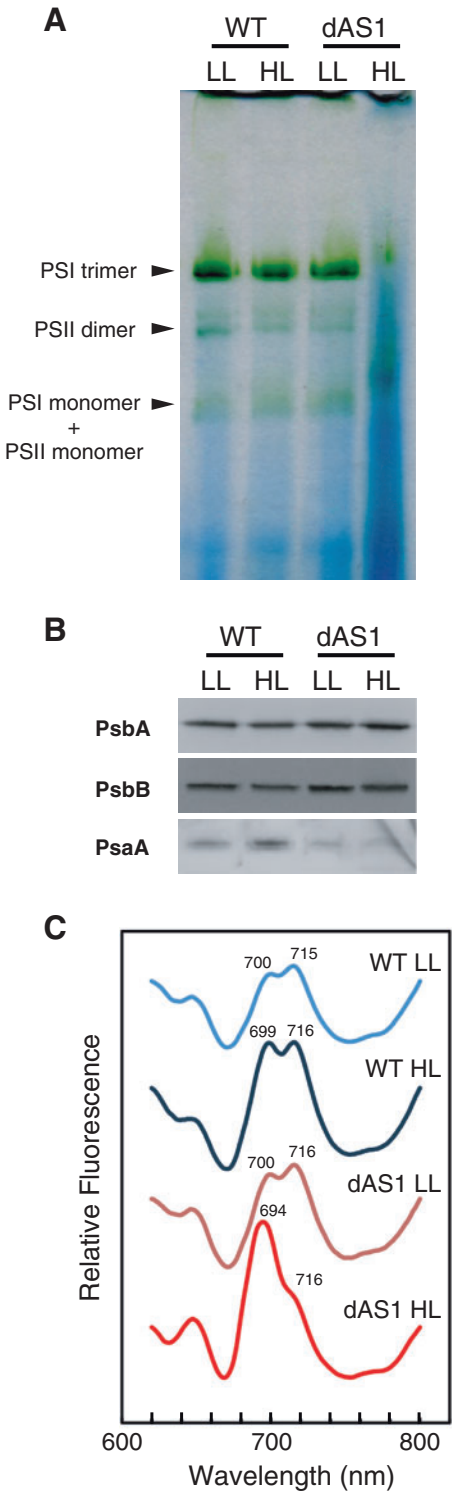

Fig. 6 Effects of light intensity on the organization and the protein levels of photosystems in the WT and dAS1. Membrane fractions were prepared from the cells grown under LL (50 μmol m^{-2} s^{-1}) or HL (400 μmol m^{-2} s^{-1}) conditions for 48 h, and solubilized with *n*-dodecyl-maltoside. (A) The samples containing 2 μg of Chl were separated by BN-PAGE. (B) The samples containing 0.2 μg of Chl were separated by SDS–PAGE in the presence of 5 M urea and analyzed by immunoblotting using antibodies against PsbA, PsbB and PsaA. (C) Low-temperature (77 K) Chl fluorescence spectra of the membrane fractions containing 5 μg ml^{-1} Chl. The excitation wavelength was set at 430 nm.

S. elongatus mutant is unable to increase its growth rate in response to an increase in light intensity (**Fig. 1**). The mutant shows enhanced sensitivity of PSII to photodamage (**Fig. 5**), indicating destabilization of PSII, and, when grown under HL conditions (400 μmol photons m^{-2} s^{-1}), shows much lower PSII activity and Chl contents as compared with the WT cells or LL-grown mutant cells (**Fig. 4**). These findings indicate the essential role of Aas in acclimation to HL conditions.

Although the molecular mechanism of PSII destabilization in the Aas-deficient mutant remains unclear, several mechanisms could account for the observed phenotype. Purified PSII preparations contain lipid molecules, at least some of which are thought to be of functional importance; PG has been shown to be essential for the activity of electron transport from Q_A to Q_B (Gombos et al. 2002). If the function of PG is lost by deacylation, the PSII reaction center would become more susceptible to photoinhibition under HL conditions. A more direct relationship between the stability of PSII and PG is to be found on the donor side of PSII; PG has been shown to be required for proper binding of the extrinsic proteins PsbO, PsbU and PsbV on the donor side of PSII to sustain a functional Mn cluster (Sakurai et al. 2007a). A similar role for DGDG in sustaining an Mn cluster has also been proposed (Sakurai et al. 2007b). Deacylation of these functionally crucial lipid molecules may destabilize the Mn cluster to render PSII highly sensitive to photodamage. Other possible mechanism(s) for the destabilization of PSII include the action of FFAs and lysolipids. As mentioned above, FFAs can serve as surfactants and hence would exert adverse effects on thylakoid membranes. Lysolipids could also act as surfactants, perturbing the membrane structure particularly in the presence of FFA (Davidsen et al. 2002). Accumulation of FFAs and lysolipids in the thylakoid membrane thus would perturb the function of the membrane and could also affect the structure of PSII, rendering it unstable. Further studies are required to elucidate the molecular mechanism(s) of PSII destabilization in the Aas-deficient mutant.

By growing a PG-less mutant of *Synechocystis* sp. strain PCC 6803 with exogenously added PG with a defined fatty acid composition, Laczko-Dobos et al. (2010) demonstrated re-tailoring of the PG molecules involving deacylation and reacylation under illumination of 30 μmol photons m^{-2} s^{-1}. By feeding the cells of *Synechocystis* sp. strain PCC 6803 and *S. elongatus* strain PCC 7942 with radiolabeled PG and SQDG, Kaczmarzyk and Fulda (2010) showed Aas-dependent channeling of the labeled fatty acids to MGDG under illumination of 38 μmol photons m^{-2} s^{-1}. In accordance with the previous reports, the dAS1 mutant cells contained larger amounts of FFAs and lysoPG than the WT cells when grown under LL conditions, confirming the occurrence of deacylation and reacylation of PG. Since the cellular content of FFAs is markedly increased by growth of the mutant under the HL conditions with a concomitant increase in the amounts of lysoPG, MGMG, DGMG and SQMG (**Fig. 2**), it is deduced that deacylation of membrane lipids is activated during growth under the HL conditions. Although it seems to be a part of the adaptive response of the cells to the HL conditions, the physiological significance of the HL-induced lipid deacylation is currently unclear,

because LL- and HL-grown WT cells show no significant difference in the composition of lipid molecular species in MGDG, DGDG and SQDG (Fig. 3). Identification and characterization of the lipases and lysolipidacyltransferases responsible for the deacylation and reacylation reactions, respectively, are needed to clarify the physiological relevance of the HL-induced lipid remodeling.

In the absence of Aas, dAS1 cells synthesize acyl-ACP solely via the fatty acid biosynthetic pathway. The accumulation of lysolipids in HL-grown dAS1 cells (Fig. 2C) indicates that the de novo synthesized acyl-ACP is insufficient to saturate the reacylation reaction of lysolipids. The decrease in the intracellular acyl-ACP level seems to account for the decrease in average chain length of the fatty-acyl moieties of membrane lipids in HL-grown dAS1 (Fig. 3); Under such conditions, de novo lipid biosynthesis as well as the reacylation reaction of lysolipids would compete strongly for acyl-ACP with the elongation reaction of acyl-ACP.

The FFA excretion system using cyanobacteria is thought to have an advantage over the triacylglycerol production system using eukaryotic algae in that recovery of the cells from the growth medium, an energy-intensive process in biofuel production, is not required. However, the yield of cyanobacteria-based production of FFA is currently too low for large-scale production (Ruffing and Jones 2012, Ruffing 2014). Since light is the sole source of energy for the photosynthetic biofuel production, it is necessary to increase the light intensity for cultivation of the cells to increase the yield. For large-scale cultivation, it is also essential to maintain a mass culture under sunlight, where the light intensity reaches $>$ 1,000 μmol photons m^{-2} s^{-1}. The light sensitivity of the *aas* mutants would severely impair the productivity of FFA under such conditions. It is hence essential to elucidate the mechanism of enhancement of the sensitivity of PSII to photoinhibition in the *aas* mutant. If FFAs are toxic, it is necessary to introduce transporter(s) that have the capacity to export FFA out of the cells to reduce the cellular FFA content. If lysolipids are toxic, it would be necessary to increase the activity of de novo synthesis of acyl-ACP to maintain its cellular content and to raise the activity of lysolipidacyltransferases to reduce the content of lysolipids. More basic research is required to improve the FFA productivity of genetically engineered cyanobacteria by solving the problem of the HL sensitivity.

Materials and Methods

Strains and growth conditions

A derivative of *S. elongatus* PCC 7942 (R2-SPc; referred to as the WT strain) which is cured of the resident small plasmid pUH24 (Kuhlemeier et al. 1983) and the *aas*-deficient mutant were grown photoautotrophically at 30°C under continuous illumination provided by a white-light-emitting diode lamp (VBL-SL150-LL, Valore) and with aeration by air supplemented with 2% (v/v) CO_2. The basal medium used was the modified BG11 medium described previously (Suzuki et al. 1995) supplemented with 15 mM KNO_3 as a nitrogen source and buffered with 20 mM HEPES-KOH (pH 8.2).

Generation of *aas*-deficient mutants

Transformation of the cyanobacteria and isolation of homozygous mutants were performed as described by Williams and Szalay (1983). A defined *S. elongatus* mutant (dAS1) deficient in the *aas* gene (synpcc7942_0918) was constructed by deleting the central portion of the gene from the genome by the marker exchange–eviction mutagenesis method (Ried and Collmer 1987) using a 3.8 kbp *nptI–sacB* cartridge excised from pRL250 (Cai and Wolk 1990) as the selection marker (Supplementary Fig. S1). In dAS1, a 662 bp internal segment of *aas*, corresponding to nucleotides 705–1,366 of the 1,950 nucleotide long coding region, had been replaced with an 18 nucleotide sequence 5′-GGATCCT CTAGAGTCGAC-3′.

FFA and lipid analysis

For analysis of FFA and lipids in cyanobacterial cells, 30 ml aliquots of the cultures were centrifuged at 1,500 × *g* for 10 min to collect the cells. The cells were resuspended in 3 ml of methanol and stored at −20°C until use. To normalize the lipid and fatty acid contents to the cell number, total lipids were extracted from an appropriate volume of the samples, corresponding to 1.25×10^9 of the cells, by a modified Folch method as follows (Folch et al. 1957, Ikeda 2015). Samples from biological triplicates were individually dried by centrifugal concentration at 4°C. Chloroform (250 μl) containing 0.8 μM each of the internal standards (d18:1/12:0 sphingomyelin and 14:0/14:0 phosphatidylcholine) and methanol (500 μl) were added to each sample and mixed vigorously to extract lipids and fatty acids. After incubation at room temperature for 15 min, 50 μl of Milli-Q water was added to each sample and the mixed suspension was further incubated for 15 min at room temperature. The samples were then centrifuged at 1,000 × *g* for 10 min at room temperature to remove the debris, and the resultant supernatant was collected in a liquid chromatography–mass specrometry (LC-MS) vial. Aliquots of 2 μl of the cyanobacterial lipid extract were analyzed by a Triple TOF 5600 System (AB SCIEX) with an Agilent 1290 Infinity LC system (Agilent Technologies) in the negative ion mode with the following parameter settings: −4.5 kV for the ion-spray voltage, 500°C for the ion-source temperature, −45 V for the collision energy of the data-dependent scan, 10 V for the collision energy spread and *m/z* 100–1,000 for the MS and MS/MS scan range (Ikeda 2015). The reversed-phase LC separation was achieved by an ACQUITY UPLC HSS column (particle size, 1.8 μm, 50 × 2.1 mm i.d., Waters Corporation) at 45°C with a gradient solvent system at a total flow rate of 300 μl min^{-1}. The mobile phase was prepared by mixing (A) acetonitrile/methanol/water (20/20/60; 5 mM ammonium formate) and (B) isopropanol (5 mM ammonium formate). The eluent composition was initially 100% A and successively changed as follows: change to (A/B: 60/40) in 5 min; change to (A/B: 36/64) in 2.5 min followed by isocratic elution for 4.5 min; change to (A/B: 17.5/82.5) in 0.5 min; change to (A/B: 15/85) in 6.5 min; change to (A/B: 5/95) in 1 min. All the changes in solvent composition were linear with respect to time. The lipid molecular species and FFAs were accurately identified by MS/MS analyses, and the MS data of the Screen-Well® Fatty Acid Library (Enzo Life Sciences). MS data processing was performed by applying the MultiQuant Software (AB SCIEX) to detect each chromatogram peak with quantitative accuracy.

For determination of the total concentration of FFAs in the external media, 0.5 ml aliquots of cultures were passed through a membrane filter (SPIN-X, Corning) and the filtrates (50 μl) were analyzed by using the Free Fatty Acid Quantification Kit (Biovision) according to the manufacturer's instruction.

Measurement of photosynthetic activity

Photosynthetic oxygen-evolving activity of the cyanobacterial cultures was measured using a Clark-type oxygen electrode (DW1, Hansatech) at 30°C under illumination at 1,000 μmol photons m^{-2} s^{-1} with or without addition of 1 mM pBQ. Photosynthetic yield of PSII was determined by measuring the F_v/F_m ratio using anAquaPen-C fluorometer (AP-C100, Photon Systems Instruments). The samples were dark-adapted for 5 min before measurement.

Preparation of membrane fractions and protein analysis

The total membrane fraction including the thylakoids was prepared as described by Aoki et al. (2014) and resuspended in a buffer containing 50 mM HEPES-KOH, pH 7.5, 10 mM $MgCl_2$, 5 mM $CaCl_2$ and 20% (w/v) glycerol. Fluorescence emission spectra of the membrane suspensions (5 μg Chl ml^{-1}) were recorded at 77 K using a fluorescence spectrophotometer (FP777w,

JASCO). The excitation wavelength was 430 nm with a bandwidth of 20 nm. The emission bandwidth was 3 nm. For BN-PAGE analysis of photosystems, the membrane samples were solubilized with 0.3% *n*-dodecylmaltoside to give a final Chl concentration of 0.3 μg ml^{-1}. BN-PAGE was performed using the NativePAGE™ Novex® 4–16% Bis-Tris Protein Gel (Life Technologies). SDS–PAGE was performed as described by Laemmli (1970). Immunoblotting was performed as described previously using antisera against PsbA, PsbB and PsaA (Kada et al. 2003).

Other methods

Chl was determined as described by Mackinney (1941). The cell number was determined by using a particle counter/analyzer (CDA-1000, sysmex). All the statistical analyses were conducted with SPSS Statistics software (IBM).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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