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The Acyl-Acyl Carrier Protein Synthetase from *Synechocystis sp.* PCC6803 mediates Fatty Acid Import

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Abstract

Transfer of fatty acids across biological membranes is a largely uncharacterized process although essential at membranes of several higher plant organelles like chloroplasts, peroxisomes or the endoplasmic reticulum. Here, we analyzed loss of function mutants of the unicellular cyanobacteria *Synechocystis* sp. PCC 6803 as a model system to circumvent redundancy problems encountered in eukaryotic organisms. Cells deficient in the only cytoplasmic *Synechocystis* acyl-acyl carrier protein synthetase (SynAas) were highly resistant to externally provided α-linolenic acid while wild-type cells bleached upon this treatment. Bleaching of wild-type cells was accompanied by a continuous increase of α-linolenic acid in total lipids while no such accumulation could be observed in SynAas deficient cells (Δsynaas). When SynAas was disrupted in the tocopherol-deficient, α-linolenic acid-hypersensitive *Synechocystis* mutant Δslr1736, double mutant cells displayed the same resistance phenotype as Δsynaas. Moreover, heterologous expression of SynAas in yeast mutants lacking the major yeast fatty acid import protein Fat1p (Δfat1) led to restoration of wild-type sensitivity against exogenous α-linolenic acid of the otherwise resistant Δfat1 mutant indicating that SynAas is functionally equivalent to Fat1p. In addition, liposome assays provided direct evidence for the ability of purified SynAas protein to mediate α-[14C]linolenic acid retrieval from preloaded liposome membranes via synthesis of [14C]linolenoyl-ACP. Taken together our data show that an acyl-activating enzyme like SynAas is necessary and sufficient to mediate transfer of fatty acids across a biological membrane.
Introduction

Fatty acids have many functions in plants for example as structural components of phospho- and galactolipids, as storage reserves in triacyl glycerols or as precursors for signaling molecules and plant hormones. As essential components of biological membranes they enable the subcellular compartmentalization of plant cells and ensure the vital separation of biochemical reactions and pathways that require different chemical environments.

Transmembrane transport of fatty acids has been characterized in detail in unicellular organisms (Black and DiRusso, 2003). In gram-negative bacteria like *Escherichia coli* two components contribute to the uptake of fatty acids from the external media. One is the integral membrane protein FadL that is localized in the outer membrane of the *E. coli* envelope and exhibits typical transport protein characteristics. Consequently, *FadL* defective mutants do not show any uptake of exogenous long-chain fatty acids (Black et al., 1985). The other component, FadD, displays acyl-CoA synthetase activity and is associated with the inner membrane of the cell envelope. Both FadL and FadD are necessary and act in concert in a process termed vectorial acylation since mutant cells lacking either activity are unable to grow on media containing long-chain fatty acids as the sole carbon source (Nunn and Simons, 1978).

In *Saccharomyces cerevisiae* a similar vectorial acylation system consisting of a transmembrane protein and acyl-CoA synthetases exists. Here both, the membrane protein Fat1p and the acyl-activating enzymes Faa1p and Faa4p are associated with the plasma membrane. Mutants lacking either Fat1p or any of Faa1 or Faa4 activity are unable to grow on long-chain fatty acids when endogenous fatty acid synthesis is inhibited through addition of cerulenin (Faergeman et al., 1997; Faergeman et al., 2001). Hence, although Fat1p harbors an acyl activating domain (Obermeyer et al., 2007), the presence of at least one of the acyl-CoA synthetases is essential for import of long-chain fatty acids into yeast cells in addition to a functional Fat1p.

In higher plants, fatty acid biosynthesis is exclusively located in chloroplasts implicating the necessity of fatty acid export from chloroplasts destined for assembly of phospholipids derived from the “eukaryotic pathway” in the endoplasmatic reticulum.
(Gardiner et al., 1982). However, while knowledge about the mechanisms involved in transfer of lipids from the chloroplast to the endoplasmic reticulum and vice versa has greatly advanced in recent years (Benning, 2008), understanding of fatty acid transport across the chloroplast membrane is still limited. Tracer experiments applying $^{18}$O or $^{14}$Cacetate to spinach or pea leaves (Pollard and Ohlrogge, 1999; Koo et al., 2004) provided initial experimental verification for a model first outlined by Shine et al. (1976). This model involves thioesterase mediated cleavage of acyl-ACP (acyl carrier protein) at the inner envelope membrane, transfer of the free fatty acids to the outer envelope membrane and acyl-activating enzyme activity at the outer envelope membrane. Moreover, incubation of isolated chloroplasts with radiolabeled fatty acids revealed that chloroplasts are also able to take up exogenously supplied fatty acids and elongate and incorporate them into lipids (Thompson et al., 1986; Koo et al., 2005). Koo et al. (2005) provided evidence that the acyl-activating enzymes AAE15 and AAE16 are essential in that process. Their study showed that isolated Arabidopsis thaliana chloroplasts activated exogenously applied laureate very rapidly through esterification to acyl-carrier protein and that this reaction is dependent mostly on AAE15.

In both E. coli and yeast, acyl-activating enzymes are involved in transmembrane fatty acid transport. Since the large superfamily of 63 acyl activating enzymes in Arabidopsis suggested possible obstruction of mutant analyses through functional redundancy (Schnurr et al., 2002; Shockey et al., 2003), we employed the more simple unicellular cyanobacteria Synechocystis sp. PCC 6803 as a model. Previous studies had revealed the presence of only a single protein in Synechocystis as homologous to higher plant acyl-activating enzymes (Kaczmarzyk and Fulda, 2010). These studies also established that the Synechocystis enzyme (Aas) specifically uses acyl carrier protein (ACP) as co-substrate to recycle free fatty acids occurring naturally in Synechocystis cells (Kaczmarzyk and Fulda, 2010). Here we provide evidence that Aas from Synechocystis (SynAas), is also involved in transfer of free fatty acids across membranes by vectorial acylation and discuss an analogous mechanism for fatty acid transfer across the chloroplast envelope.
Results

We used the deduced amino acid sequence of *Arabidopsis* LACS9 to search for homologous proteins in the cyanobacteria *Synechocystis* sp. PCC 6803. Analogously to Kazmarczyk and Fulda (2010), we identified Slr1609 as the only homologous protein through the BLASTP function of the CyanoBase website (http://genome.kazusa.or.jp/cyanobase). Slr1609 had been named Aas (SynAas throughout this manuscript). In order to gain additional insight into SynAas function in *Synechocystis* metabolism, we generated knockout mutants (Δsynaas) through homologous recombination (Supplemental Figure 1) and analyzed cell growth and viability in various conditions.

**Δsynaas cells are resistant to α-linolenic acid treatment**

On regular BG11 medium, fully segregated Δsynaas mutants did not display any phenotypic deviation from wild-type cells (Figure 1), indicating that SynAas function is not essential under the used conditions. Since previous studies had shown that exogenous α-linolenic acid, once taken up into the cell, has a detrimental effect on cells (Maeda et al., 2005; Kunz et al., 2009), we tested cell growth and viability of Δsynaas when exposed to increasing concentrations in the growth media. Interestingly, *Synechocystis* wild-type cells were impaired in growth both on plates and in liquid culture supplemented with α-linolenic acid (Figure 1). Whereas plate drop assays showed that wild-type cells when diluted from a pre-grown culture on control media simply would not grow in the presence of 10 µM α-linolenic acid or above, the phenotype in liquid culture was much more dramatic. Here, an initially green culture diluted to an OD750 of 0.25 (compare 0 µM α-linolenic acid in Figure 1B) would completely bleach within 24 h when challenged with 20 µM α-linolenic acid or above (Figure 1B). Moreover, when monitoring growth of wild-type α-linolenic acid challenged cultures as optical density, cell growth rapidly ceased and cells died (Figure 1C). In contrast Δsynaas mutant cells were unaffected on plates, showed resistance in liquid culture to very high concentrations of α-linolenic acid of up to 200 µM and were able to maintain growth rates comparable to control conditions at 40 µM α-linolenic acid (Figure 1B, C). These data show that high concentrations of α-linolenic acid in the external media have a detrimental effect on
Synechocystis cells and that this effect is dependent on the intracellular, SynAas-dependent activation of fatty acids.

**Photosynthesis is impaired in Synechocystis wild-type but not Δsynaas cells**

Since α-linolenic acid-challenged wild-type cells bleached completely after 24 h of incubation, we investigated the impact of exogenously fed α-linolenic acid on photosynthesis in wild-type and Δsynaas cells after shorter periods of time using chlorophyll fluorescence. We monitored the photosynthetic electron transport rate (ETR), as a sensitive parameter for intactness of the photosynthetic apparatus. The ETR in wild-type cells started to steeply decrease after 5 h of incubation with 150 µM α-linolenic acid until virtually no photosynthetic electron transport was detectable after 9 - 10 h of incubation (Figure 2). However, the wild-type cell culture did not visibly bleach in these conditions as after 24 h of α-linolenic acid treatment (Figure 1B). In contrast, photosynthesis in Δsynaas cells remained unchanged in response to α-linolenic acid incubation (Figure 2). In addition control treatment with only the α-linolenic acid solvent (ethanol) had no effect on ETR in wild-type or Δsynaas cells (Figure 2).

**Subcellular compartmentation is lost in Synechocystis cells in response to α-linolenic acid treatment**

We analyzed the impact of α-linolenic acid treatment on cell structure and integrity in wild-type and Δsynaas cells using electron microscopy. Samples taken after 0, 6 and 10 h of incubation with 150 µM α-linolenic acid showed a gradual change in cell structure with duration of α-linolenic acid treatment in wild-type cells. Here, an accumulation of electron-dense particles could be observed after 6 h (Figure 3). In electron microscopy, lipophilic structures often appear as dark regions (Neiss, 1983). Hence, the detected electron-dense particles could be assigned either to collapsed membrane material or accumulation of free fatty acids and lipids. These particles increased massively in size and number after 10 h of incubation and the subcellular compartmentation into peripheral thylakoid membranes and central carboxysome and DNA was almost completely lost (Figure 3), congruent with the observed lack of photosynthetic electron transport in these cells (Figure 2). In contrast Δsynaas cells did not show any alteration of their ultrastructural appearance after 6 or 10 h of incubation (Figure 3).
**Wild-type but not Asynaas cells accumulate α-linolenic acid in short and long-term incubation experiments**

In order to test whether exogenously fed α-linolenic acid accumulates in *Synechocystis* cells, we exposed wild-type and *Asynaas* cells to moderate concentrations (33 µM) of radiolabeled α-[14C]linolenic acid for up to 60 minutes and measured cell-associated radioactivity after intensive washing. Data show that even immediately after addition of α-[14C]linolenic acid a basal level of radioactivity remained attached to the cells and could not be washed off from either wild-type or *Asynaas* (Figure 4A, time point 0). However, while this basal level remained constant in *Asynaas* cells after 10 min of incubation and was significantly less than in wild-type cells at any time point, cell-associated radiolabel constantly increased in wild-type cells indicating continuous uptake and incorporation (Figure 4A).

Moreover, when determining the fatty acid composition in total lipid extracts of *Synechocystis* cells treated for up to 10 h with α-linolenic acid (150 µM), we found that the α-linolenic acid concentration strongly increased in wild-type cells to values of up to 67.4 ± 8.6 nmol • mg⁻¹ DW, where the concentration in untreated cells was 1.9 ± 0.4 nmol • mg⁻¹ DW (Figure 4B). In contrast, no continuous increase of α-linolenic acid in total lipids could be detected in *Asynaas* cells. There is, however, as with the radiolabeled α-linolenic acid treatment, an initial leap to concentrations significantly above the level in untreated cells that during the further course of experiments remained constant (Figure 4B, C). This initial increase of α-linolenic acid in *Asynaas* cells probably results from free fatty acids that are adhering to the cell surface or have been integrated into the plasma membrane and not been washed off during wash steps after incubation.

The increase in α-linolenic acid in wild-type cells over the course of 10 h was specific for this fatty acid, since the other fatty acids did not display any change in levels (Figure 4C).

**Exogenous α-linolenic acid accumulates in lipids and as free fatty acid**

In order to assess the distribution of α-linolenic acid absorbed from the external media among cellular lipid species we applied thin layer chromatography. Lipids were extracted from cell samples after 0, 6 and 10 h of α-linolenic acid incubation and separated by thin layer chromatography. A substantial increase of free α-linolenic acid is visible in the
wild-type strain after 6 and 10 hours of incubation (Figure 5) indicating that free fatty acids constitute a major component of the electron dense accumulations apparent in the electron micrographs (Figure 5). Additionally there is an increase in monogalactosyl diacylglycerol (MGDG), sulfoquinovosyl diacylglycerol (SQDG) and phosphatidyl glycerol (PG) over time. When applying $\alpha$-$[^{14}\text{C}]$linolenic acid for incubation times of up to 60 minutes radio label could clearly be detected in major lipid classes after 20 minutes (Supplemental Figure 2) showing that $\alpha$-linolenic acid is incorporated into most of the predominant lipid species of *Synechocystis* (Wada and Murata, 1998). We suspect that the additional band, appearing right above MGDG (Figure 5), represents an MGDG with two 18:3 fatty acid residues, which would lead to a more lipophilic molecule than the common MGDG with 16:0 fatty acids at the sn-2 position (Wada and Murata, 1990). An unknown very polar molecule, running underneath phosphatidyl glycerol (PG) is also increasing over time. However, we could not clarify its identity with our standards. In contrast to the findings in wild type, there is no increase in any of the lipid species in the Δsynaas mutant (Figure 5).

**α-linolenic acid-hypersensitivity of tocopherol deficient Δslr1736 cells is reverted to resistance of Δsynaas cells in Δslr1736/Δsynaas double mutants**

For cyanobacteria it has been shown that $\alpha$-tocopherol is essential for diminishing lipid peroxidation caused through $\alpha$-linolenic acid since $\alpha$-tocopherol-free Δslr1736 mutants are hypersensitive to treatment with exogenous $\alpha$-linolenic acid (Maeda et al., 2005). By generating and analyzing cell growth of a Δslr1736/Δsynaas double mutant in the presence or absence of $\alpha$-linolenic acid we intended to determine whether or not $\alpha$-linolenic acid enters *Synechocystis* cells. Plate drop assays showed that all cell lines grew equally well on control media but that $\alpha$-tocopherol-free Δslr1736 cells were unable to grow on plates containing as little as 5 μM $\alpha$-linolenic acid, a concentration that did not affect growth of *Synechocystis* wild-type cells (Figure 1A, Figure 6, Supplemental Figure 4). In addition Δslr1736 growth in liquid culture was as severely impaired in the presence of $\alpha$-linolenic acid as wild-type cells (Figure 1C). In contrast, the Δslr1736/Δsynaas double mutant grew not only at concentrations that were still tolerated by wild-type cells but also at much higher concentrations that are tolerated by Δsynaas
mutants only (Figure 1C, Figure 6, Supplemental Figure 3). These data strongly indicate that exogenous α-linolenic acid is not executing its detrimental effect in Δslr1736/Δsynaas double and Δsynaas single mutants because it is not entering these cells.

*Overexpression of SynAas in yeast wild-type or Δfat1 mutant cells leads to increased α-linolenic acid sensitivity*

The components involved in fatty acid import in *Saccharomyces cerevisiae* have been identified as Fat1p, Faa1p and Faa4p, where Fat1p is the essential integral membrane transport protein. Postulating that fatty acid import-defective yeast mutants may also be resistant to the toxic effects of exogenous α-linolenic acid, we analyzed single mutant growth in the presence of high external concentrations of α-linolenic acid. Wild-type yeast cells challenged with 3.6 mM α-linolenic acid in drop assay experiments were unable to grow in the absence of any additional carbon source (data not shown), however, reduced growth of wild-type cells compared to control plates could be observed when glucose was added at a low concentration (0.55 mM, Figure 7B). In contrast Δfat1 single mutant cells showed α-linolenic acid-resistant growth on the same plates (Figure 7B). Since yeast cell growth in liquid culture measured as OD 600 could more reliably be characterized at a glucose concentration of 5.5 mM in the media we determined the effect of α-linolenic acid on wild-type and Δfat1 cells at moderate glucose concentrations (Figure 7A). Under these conditions wild-type cell growth in the presence of 3.6 mM α-linolenic acid is only weakly inhibited compared to control conditions and does not differ from Δfat1 growth (Figure 7A). However, overexpression of a SynAas-GFP fusion protein in the Δfat1 mutant and wild-type background lead to a significant depression of cell growth (Figure 7A) which was even more evident in the presence of low glucose in the media (Figure 7B). Here, the residual growth of SynAas-GFP expressing wild-type cells observed in liquid culture (Figure 7A) was completely abolished in SynAas-GFP over-expressing cells (Figure 7B). These data support the conclusion that the GFP-tagged SynAas protein can functionally complement Fat1p-mediated α-linolenic acid uptake and that an acyl-activating enzyme alone is sufficient to mediate import of fatty acids from the external media into the cell.
**SynAas can retrieve and activate α-linolenic acid from artificial liposome membranes**

In order to analyze the ability of SynAas to retrieve and activate fatty acids from artificial membranes, we labeled large unilamellar vesicles (0.2 µm) with α-[14C]linolenic acid and incubated these liposomes with purified SynAas. When α-linolenoyl-ACP was determined as water-soluble radioactivity in the supernatant of [14C]-labeled liposomes, substantial synthesis of α-linolenoyl-ACP was detected in assay conditions containing SynAas, ACP, ATP and Mg^{2+} (Figure 8A). A low, about four times reduced level of radioactivity was observed in the absence of either ATP (with SynAas and ACP present, ‘no ATP’ in Figure 8A) or SynAas and ACP (with ATP present, ‘buffer only’ in Figure 8A) or when adding boiling-inactivated SynAas to otherwise complete assay media (‘boiled SynAas in Figure 8A). Complementary, the [14C]-label was lowest in assay conditions providing all components and approximately equally high when carried out without either ATP or SynAas and ACP or with boiling-inactivated SynAas when liposome-associated radioactivity was determined from the liposome pellet after assay performance (Figure 8B). This indicates that α-[14C]linolenic acid could be retrieved and activated from liposome membranes through action of SynAas.

**Discussion**

Although polyunsaturated fatty acids are part of all plant membranes, they are toxic to the cell in higher concentrations. In plants, accumulation of α-linolenic acid within the cell is detrimental to the integrity of chloroplasts by unfolding of grana stacks and subsequent changes in the thylakoid membrane ultra structure (Siegenthaler, 1972; Okamoto et al., 1977; Kunz et al., 2009). Here we used the cytotoxic effect of intracellularly accumulating α-linolenic acid as a tool to demonstrate the involvement of an acyl activating enzyme in fatty acid translocation across biological membranes.

**Loss of function of the acyl-activating enzyme protects the cell from the toxic effect of α-linolenic acid**

Analyses of *Synechocystis* wild-type cells challenged with α-linolenic acid all revealed negative effects on cell viability detected as impaired growth and bleaching (Figure 1), reduced photosynthetic electron transport (Figure 2), disintegration of membranes and
subcellular organization and agglomeration of large lipophilic structures inside cells (Figure 3). Simultaneous with this negative impact on wild-type cells the content of α-linolenic acid in total lipids and as free fatty acid steadily increased with duration of treatment (Figure 4 B and C, Figure 5). The apparently continuing, ATP-consuming activation of α-linolenic acid in the presence of deteriorating photosynthesis might be fueled for a limited period of time through breakdown of storage reserves like glycogen. Since a negative impact of α-linolenic acid on ultrastructure and photosynthetic electron transport has also been described for isolated chloroplasts (Siegenthaler, 1972; Okamoto et al., 1977; Golbeck et al., 1980; Kunz et al., 2009) it appears reasonable that the accumulation of excess amounts of α-linolenic acid within Synechocystis cells is the major cause for the observed toxicity. No accumulation of α-linolenic acid could be observed in Δsynaas mutant cells, neither in short- nor in long-term experiments (Figure 4, 5). Consequently, α-linolenic acid resistant growth could be observed in the non-accumulating Δsynaas mutant cells (Figure 1, 4). The exact mechanism of α-linolenic acid toxicity to cells remains unclear. Although our study did not aim at exploring the physiological basis for the observed toxicity in Synechocystis and yeast wild-type cells, we adopt its occurrence as an argument for strongly reduced or absent α-linolenic acid uptake into mutant cells. In fact, our analyses of Synechocystis wild-type cells showed strongly elevated levels of α-linolenic acid in lipids and as free fatty acid (Figure 5). Since it had been shown previously that hydrogen atoms adjacent to olefinic bonds such as those found in polyunsaturated fatty acids are susceptible to oxidative attack (Singh et al., 2002), we speculated that the observed detrimental effects of α-linolenic acid might partly be caused by lipid peroxidation. Intracellular lipid peroxidation results in secondary effects like protein modification and degradation and DNA damage (McIntyre et al., 1999) which would fit well with the observed general reduction in cell viability of α-linolenic acid accumulating wild-type cells. In order to protect polyunsaturated fatty acid acyl chains from oxidative damage, cyanobacteria, algae and higher plants have evolved a scavenger mechanism employing α-tocopherol as antioxidant (Maeda and DellaPenna, 2007; Hunter and Cahoon, 2007). α-Tocopherol is a lipophilic compound that has the
unique ability to prevent oxidative damage to polyunsaturated fatty acid acyl chains through interception of fatty acid peroxy radicals and hence interruption of the chain reaction process inherent to lipid peroxidation (Schneider, 2005). Moreover, recent studies on *Synechocystis* mutants suggest that α-tocopherol is also important for repair of photodamaged photosystem II (PSII) by protecting *de novo* biosynthesis of high-turnover PSII components like D1 (Inoue et al., 2011). The tocopherol-deficient *Synechocystis* mutant Δslr1736 is highly susceptible to treatment with α-linolenic acid (Maeda et al., 2005). In agreement with the findings described by Maeda et al. (2005), we could also demonstrate that low concentrations of α-linolenic acid (5 µM) in highlight conditions (300 µE) are toxic for Δslr1736 single mutant cells (Figure 6). Interestingly, the Δslr1736 hypersensitivity phenotype could be completely reverted to Δsynaas resistance by generating a Δsynaas/Δslr1736 double knock-out (Figure 6, Supplemental Figure 3). Apparently, α-linolenic acid incorporated from the external media leads to lipid-peroxidation within *Synechocystis* cells during highlight exposure that cannot be prevented in the Δslr1736 single mutant because of the absence of tocopherols. In wild-type cells α-tocopherol is able to efficiently protect cells at 5 µM α-linolenic acid but evidently insufficient for cells to tolerate an external α-linolenic acid concentration of 10 µM and above. In contrast Δsynaas/Δslr1736 double mutants are as resistant as Δsynaas single mutants to α-linolenic acid of 10 µM and above (Figure 1C, Figure 6, Supplemental Figure 3). Because α-tocopherol exhibits its antioxidant function inside cells the absence of α-linolenic acid-sensitive growth in both Δsynaas/Δslr1736 and Δsynaas mutant can only be explained by a strongly reduced or even absent uptake of the exogenously supplied α-linolenic acid, which is well supported by the α-linolenic acid non-accumulating phenotype of Δsynaas cells (Figure 4).

Taken together, these results suggest that the uptake mechanism in *Synechocystis* is strictly coupled to fatty acid activation. This adds a function in fatty acid uptake for SynAas to its role in internal fatty acid recycling demonstrated by Kaczmarzyk and Fulda (2010) who had concluded from their results that fatty acid uptake is independent from SynAas.

Consequently fatty acids can only enter the cell by activation with ACP. Since wild-type
cells constantly import fatty acids under the imposed experimental conditions (Figure 4), there must be a fast recycling of ACP. In order to maintain the free ACP pool these activated fatty acids need to be rapidly integrated into lipids as demonstrated by the increase in MGDG, SQDG and PG in wild-type cells (Figure 5, Supplemental Figure 2). However, the simultaneous rise in free ω-linolenic acid in wild-type cells (Figure 5) also suggests that a substantial amount ω-linolenic acid is cleaved off from lipids. Free fatty acids accumulating in lipoid bodies as observed in electron micrographs (Figure 3) might be inaccessible for further activation by SynAas. Hence, lipoid body formation may counteract a futile cycle of repeated fatty acid activation, integration into lipids and phospholipase-mediated cleavage thereby enabling continuous influx of external fatty acids (Figure 4). Imposing a distorted fatty acid composition on Synechocystis membrane lipids might lead to the necessity of remodeling these lipids to maintain favorable physicochemical membrane properties (Okazaki et al., 2006) and may also contribute to the observed ω-linolenic acid toxicity. The rise in intracellular free fatty acids could be the result of increased phospholipase activity as a reaction to the elevated ω-linolenic acid content in polar lipids. Previous studies have revealed the natural occurrence of free fatty acids in Synechocystis in the apparent absence of an acyl-ACP thioesterase (Kaczmarzyk and Fulda, 2010). Thus lipase activity appears as a reasonable explanation for free fatty acids inside Synechocystis cells. A functional β-oxidation pathway appears to be missing in cyanobacteria. Therefore incorporation of fatty acids into lipids is the only destination for moderate amounts of imported fatty acids. Excess fatty acids are apparently secreted into the surrounding media since Δsynaas alone and in combination with other genetic modifications was recently shown to cause fatty acid enrichment in the culture medium (Kaczmarzyk and Fulda, 2010; Liu et al., 2011). In this context the discovery of the fatty acid resistance phenotype in Δsynaas may prove useful for the production of biofuels in approaches aiming at engineering organisms that accumulate high concentrations of fatty acids in the culture media.
SynAas mediates fatty acid import when heterologously expressed in Saccharomyces cerevisiae

In order to further examine the involvement of acyl activating enzymes in the import of fatty acids, we tested the Saccharomyces cerevisiae mutant Δfat1 upon treatment with α-linolenic acid. Fat1p, as the postulated fatty acid transporter, acts in concert with Faa1p and Faa4p in vectorial acylation in Saccharomyces cerevisiae (Obermeyer et al., 2007). Analysis of the Δfat1 mutant had revealed that the import of oleate or the fatty acid analogon BODIPY-3823 is strongly reduced (Faergeman et al., 1997). However, earlier studies also revealed that the two acyl-activating enzymes Faa1p and Faa4p are essential for uptake of fatty acids (Knoll et al., 1995). Here, we describe a new phenotype of the Δfat1 mutant, which displays α-linolenic acid-resistant growth on plates containing high concentrations of α-linolenic acid, whereas Saccharomyces cerevisiae wild-type cells are highly sensitive under these conditions (Figure 7). We propose that, as in Synechocystis, the reduced uptake of toxic α-linolenic acid is the major cause for the resistance phenotype.

In our studies, we focused on Fat1p because it is the only acyl-activating enzyme with several membrane spans, characteristics of a classical transport protein, and has also been proven to have a major impact on fatty acid import in yeast (Faergeman et al., 1997). Overexpression of the cyanobacterial SynAas protein fused to GFP in the Δfat1 mutant lead to an increased sensitivity when grown in media containing 3.6 mM α-linolenic acid and 5.5 mM glucose (Figure 7A). More strikingly, α-linolenic acid hypersensitivity was imposed in wild-type cells over-expressing SynAas-GFP upon α-linolenic acid treatment (Figure 7). We postulate that in addition to the functional, endogenous import mechanism of wild-type cells, SynAas mediates increased import of toxic fatty acids, which causes the observed growth retardation. A generally stronger α-linolenic acid sensitivity in SynAas-expressing wild-type and Δfat1 mutants could be observed by reducing the glucose content in the media to 0.55 mM (Figure 7B). This effect probably reflects the fact that the uptake of fatty acids becomes less important the more glucose is provided as a carbon source and implies that the native yeast fatty acid import mechanism may be
down-regulated at the transcriptional or post-transcriptional level in the presence of sufficient quantities of glucose.

The recovery of the Δfat1/SynAas α-linolenic acid-sensitive phenotype and the hypersensitive phenotype of the WT/SynAas cells suggests that the acyl-activating enzyme from Synechocystis is functionally equivalent to Fat1p and further strengthens our assumption that SynAas is able to mediate fatty acid import without the help of any additional transport protein.

**Fatty acids embedded in membranes serve as substrate for the acyl-activating enzyme**

It had been demonstrated previously that uncharged fatty acids can rapidly flip-flop between the exoplasmic face and the cytoplasmic face of membranes without the help of proteins (Kamp and Hamilton, 1992). In order to generate an influx of fatty acids across membranes, a mechanism needs to be present that removes fatty acids from the membrane at the cytosolic side. Flipping of free fatty acids from one leaflet to the other is reversible, however, when fatty acids are removed on one side, a continuous, trans-membrane flux of fatty acids would be created. Such a mechanism can be driven by an intracellular acyl-activating enzyme, which retrieves the lipophilic free fatty acid from the membrane and converts it to the water-soluble acyl-ACP or acyl-CoA ester. This so-called vectorial acylation has been discussed and investigated in great detail for *S. cerevisiae* and *E. coli* as the main mediator of cellular fatty acid uptake (Black and DiRusso, 2003; Black and DiRusso, 2007). For this mechanism to work properly it would be beneficial if the acyl-activating enzyme was membrane associated. Although SynAas does not contain any apparent membrane domains according to various prediction programs, it has been purified from the membrane fraction of heterologous expression systems in this study and previous reports (Kaczmarzyk and Fulda, 2010). Moreover, SynAas has been identified in a proteomics study of Synechocystis plasma membrane proteins (Pisareva et al., 2007) indicating that it is probably membrane associated *in vivo*. In order to analyze whether an acyl-activating enzyme can process free fatty acids from artificial membranes, we established an acyl-ACP synthetase assay where the fatty acid substrate is embedded in an artificial liposome membrane. Using this assay we could show that the recombinant acyl-activating enzyme from Synechocystis is able to highly
increase the concentration of water-soluble α-linolenoyl-ACP to 79.8±13.3 nmol/l SynAas, whereas without ATP in the assay the concentration remains at 24.7±3.8 nmol/l SynAas in the supernatant (Figure 8). Concomitantly the amount of membrane embedded free fatty acids is decreasing from 417.8±33.0 nmol/l to 387.0±21.7 nmol/l in an ATP-dependent manner (Figure 8). From these results it is evident that fatty acids embedded in artificial liposome membranes can serve as substrate for an acyl-activating enzyme like SynAas and hence be transferred across a biological membrane solely by the action of such an enzyme.

A model for fatty acid translocation across membranes

Through analyses of *Synechocystis* wild-type and mutant cells, complementation of a yeast fatty acid transporter mutant and liposome experiments, we provide direct evidence for a model in which fatty acids can cross a membrane bilayer by first integrating into the membrane according to their physicochemical properties and subsequent retrieval through the action of SynAas at the cytosolic side (Figure 9). Although this model has been derived from studying cellular fatty acid uptake in a prokaryotic organism, it might be speculated that an analogous mechanism is operating at endomembranes of eukaryotic cells. Transferring this model to fatty acid export from chloroplasts of higher plants would implicate that fatty acids synthesized as acyl-ACP esters inside chloroplasts, would have to be released as free fatty acids into the inner envelope membrane by the action of a stroma localized thioesterase such as FatA1 or FatB1 (Jones et al., 1995). In the intermembrane space, an acyl-activating enzyme would activate the free fatty acid through esterification to coenzyme-A, providing fast export rates by vectorial acylation. The acyl moiety might then either be directly incorporated into phosphatidylcholine via acyl editing as recently suggested (Tjellström et al., 2012) or transferred by a second vectorial-acylation step to the cytosol for incorporation into ER derived lipids. The identity of many of the molecular components involved in this process in higher plants is still elusive. However, the *Synechocystis* and yeast mutant phenotypes reported here may prove to be useful in future research aiming to demonstrate the putative involvement of candidate proteins in fatty acid export from chloroplasts.
Materials and Methods

Strains and growth conditions

*Synechocystis* sp. PCC6803 wildtype and mutant strains were grown under constant light conditions at 45 μE m⁻² s⁻¹ and 30°C. Liquid cultures were grown in BG11 media (Rippka et al., 1979), mutant strains were cultivated in the presence of antibiotics for selection (25 μg ml⁻¹ kanamycin or 25 μg ml⁻¹ spectinomycin). *Sacharomyces cerevisiae* wild type and mutant strains were cultivated in liquid SD media (0.67% yeast nitrogen base, 2% glucose and amino acids with uracil for wildtype and without uracil for transformation selection) at 29°C.

Generation of *Synechocystis* sp. PCC6803 knockout mutants

*Synechocystis* sp. 6803 mutants were generated as follows. 710 bp of the open reading frame of SynAas were amplified from wild type genomic DNA via primers SynAas_s ctagatggcgaacacctgac and SynAas_as atgagagtttccagtctgccc. The PCR product was ligated into pGEM-T Easy (Promega, Madison, USA) to yield SynAas_pGEM-T. The HincII liberated kanamycin cassette from pUC4_Kan (GenBank accession: X06404) was inserted into *SmaI* digested SynAas_pGEM-T yielding a kanamycin resistance cassette flanked by two equal halves of the SynAas specific sequence (pGEM-T-Δsynaas_Kan). Transformation of *Synechocystis* sp. PCC 6803 with pGEM-T-Δsynaas_Kan resulted in the Δsynaas mutant strain.

SLR1736 knock-out cells were created as described before (Savidge et al., 2002): the SLR1736 open reading frame was amplified from genomic DNA using primers with added NdeI and BamHI (underlined) restriction sites: SLR1736_s tattcatatggcaactatccagaagtttga and SLR1736_as ggatcttaattgaagaagataactaaatgtc. Digestion of pGEM-T with EcoRV and NdeI offered suitable ligation sites for the NdeI, BamHI digested PCR product. pGEM-T-slr1736 was digested with *MfeI* and ligated with the *EcoRI*-digested spectinomycin resistance cassette from PUC4_S (pUC 4 with inserted spectinomycin cassette). Transformation of *Synechocystis* sp. PCC6803 and Δsynaas mutant cells with the pGEM-T-Δslr1736_Spec vector resulted in Δslr1736 and Δsynaas/Δslr1736 double mutant strains, respectively.
**Generation of Sacharomyces cerevisiae complementation strains**

The coding sequence of SynAas was amplified using the Primer SynAas_CDS_s: ctgtgggaatccctctacga and SynAas_CDS_as: aaacatttcgtcaattaaatgttg without stop codon from *Synechocystis* sp. PCC 6803 genomic DNA. By using the vector pENTR/D-TOPO (Invitrogen, Carlsbad, USA) the coding sequence of SynAas was inserted into the yeast expression vector pDR-GW-eGFP (Loqué et al., 2007) which allows for the synthesis of a C-terminal eGFP fusion protein via Gateway cloning (Invitrogen, Carlsbad, USA). Transformation of pDR-GW-SynAas-eGFP into the *S. cerevisiae* wild-type strain BY4741 and Δfat1 (YBR041w) (Euroscarf, Frankfurt, Germany) yielded the strains wild type/SynAas and Δfat1/SynAas.

**Lipid composition analysis**

Liquid culture of *Synechocystis* sp. PCC6803 wildtype and Δsynaas cells were adjusted to an OD_{750} of 0.25 and incubated with 150 µM α-linolenic acid (Sigma Aldrich, St. Louis, USA) for 10 h at 45µE m^{-2} s^{-1} and 30°C. 15 ml samples were taken every 2 h. Cells were pelleted at 10000 g and subsequently washed 2x in BG11 (10 ml) supplemented with BSA (1 % w/v) and 1x in BG11 (10 ml). Lipids and fatty acids were extracted with dichloromethane/methanol (2:1 v/v) as described in von Elert and Stampfl (2000). Analyses were performed on an Agilent 6890N GC system equipped with a flame ionization detector and helium as carrier gas in a capillary GC column (J&W DB-225, 30 m, ID 0.25 mm, film thickness 0.25 µm, Agilent, Santa Clara, USA). FAMEs were identified by comparison of retention times to FAME standards (Sigma-Aldrich, St. Louis, USA). Quantification of the fatty acids was done by comparison with internal standards (C17:0 and C23:0 methyl esters).

**Thin layer chromatography**

*Synechocystis* wild-type and Δsynaas suspension culture solutions were adjusted to OD_{750} 0.25 and incubated with 150 µM α-linolenic acid. Lipids were extracted from 50 ml of suspension culture at the time points indicated according to Bligh and Dyer (1959). Using the Linomat 5 (CAMAG, Berlin, Germany), 30 µl of the lipid extract were applied to a 20 × 10 cm high performance thin layer chromatography (HPTLC) Silica Gel 60 plate.
(Merck), which was pre-washed twice with chloroform/methanol 1:1 (v/v) and air-dried for 30 min. Lipids were separated using acetone:toluene:H₂O (91:30:8 v/v/v) as solvent. For detection of lipid bands, the TLC plate was dipped into a phosphoric acid/copper sulfate reagent (15.6 g of CuSO₄(H₂O)₅ and 9.4 ml of H₃PO₄ (85 %, w/v) in 100 ml of water) and charred at 180 °C for 10 min (Yao and Rastetter, 1985). Lipids were identified by comparison with standards of MGDG, SQDG, DGDG, PG purchased from Lipid Products Ltd, England.

**Short-term incorporation of [1-¹⁴C]-labeled α-linolenic acid**

*Synechocystis* sp. PCC6803 wild type and Δsynaas were adjusted to OD₇₅₀ 3.0 in 300 µl and incubated with 33 µM final concentration α-linolenic acid in a 1:10 dilution with [¹⁴C]linolenic acid (Hartmann Analytic) at 30°C in the light. 60 µl samples were collected every 10 min and spotted on Millipore 45 µm membrane filters. Cells were washed with 10 ml ice cold BG11 medium supplemented with 33 µM unlabeled α-linolenic acid. Radioactivity in retained cells was determined in a Beckmann scintillation counter.

**Determination of electron transport rate in Synechocystis cells**

*Synechocystis* sp. PCC6803 wild-type and Δsynaas cells were adjusted to OD₇₅₀ 0.25 and incubated with 150 µM α-linolenic acid (constant light; 45 µE m⁻² s⁻¹, 30°C). Mock treated cells were incubated with 0.1% ethanol. The photosynthetic electron transport rate was measured every 60 min using a WATER-PAM chlorophyll fluorometer (WALZ, Effeltrich, Germany). For measurements, cultures were diluted 1:10 in BG-11 medium in a final volume of 2 mL. Light curves were recorded with manufacturers standard settings with increasing photosynthetic active radiation (PAR: 380-710 nm) from 0 - 1650 (photon flux density: µmol photons m⁻² s⁻¹). The equation for the calculation of ETR is ETR=Yield x PAR x 0.5 x 0.84. Graphs display the electron transport rate at PAR 200.

**Electron microscopy**

Wild type and Δsynaas *Synechocystis* sp. PCC6803 cultures were grown to an OD₇₅₀ of 0.25, incubated with 150 µM α-linolenic acid and samples pelleted at the indicated time...
points. Cell pellets were fixed with 2% osmoniumtetroxid and 2.5% glutaraldehyde, dehydrated in an ethanol series and embedded in Epon 812 resin. Ultrathin sections were prepared using an ultramicrotome (UC7, Leica, Wetzlar, Germany). The sections were stained with lead citrate and uranyl acetat and viewed with a Phillips CM 10.

**Growth assays on plates and liquid culture**

**Synechocystis** sp. PCC6803 drop test: Liquid culture of *Synechocystis* sp.6803 wild type and the corresponding mutants were adjusted to OD<sub>750</sub> 0.05 and 5 µl was spotted in four dilutions on BG11 agar plates supplemented with 0.1% EtOH and 0-10 µM linolenic acid. Photographs were taken after 7 days of growth at constant light (45 µE m<sup>-2</sup> s<sup>-1</sup>) and 30°C. For the drop test of the tocopherol deficient mutant wild type, Δslr1736, Δsynaas/Δslr1736 and Δsynaas cultures where grown under low light conditions (15 µE m<sup>-2</sup> s<sup>-1</sup>), diluted to the indicated OD<sub>750</sub> and shifted to high light conditions (300 µE m<sup>-2</sup> s<sup>-1</sup>) (Maeda et al., 2005) and where grown for 7 days. For determination of growth rates in the presence and absence of α-linolenic acid, cultures were adjusted to an OD<sub>750</sub> of 0.25. Culture volumes of 25 ml were supplemented with α-linolenic acid (40 µM final conc.) or EtOH as control. Cultures were incubated at 30°C and medium light conditions (100 µE m<sup>-2</sup> s<sup>-1</sup>) for 48 hours. OD<sub>750</sub> were measured every 6 hours.

**S. cerevisiae** drop test: Liquid culture of *S. cerevisiae* wild type and indicated mutant strains were diluted to OD<sub>600</sub> 0.05 and 2 µl were dropped on SD Plates containing 1% tergitol (added to increase α-linolenic acid solubility), 3.6 mM (0.1 % w/v) α-linolenic acid and 0.55 mM (0.01 % w/v) or 5.5 mM (0.1 % w/v) glucose in the indicated combinations. Plates were grown for 5 days at 30°C. For the OD measurements, YNB media was prepared containing 1% tergitol, 3.6 mM (0.1 % w/v) α-linolenic acid and 5.5 mM (0.1 % w/v) glucose in the indicated combinations. The culture was continuously shaken at 29°C and OD was measured at 600 nm at the indicated time points.

**Purification of SynAas from Synechocystis and ACP from E.coli**

For amplification of the SLR1609 coding sequence from *Synechocystis* the primers SLR1609cds fw: CACCAAAATGTCTGACAGTGGCCATGGCGC and SLR1609cds rev: AAACATTTTCGTCAATTAATGTTG and *Synechocystis* genomic DNA as
template were used. Via the vector pENTR-D-TOPO (Invitrogen, Carlsbad, USA) the SLR1609 CDS were transferred to the yeast expression vector pYES-DEST52 (Invitrogen, Carlsbad, USA) to produce SLR1609 with a C-terminal 6x His tag. For amplification of the Acyl Carrier Protein coding sequence from E. coli genomic DNA the following primer combination was used: ACP_fw CACCATGAGCACTATCGAAGAACGCG and ACP_rev CGCCTGGTGCCGTTGTGTAATCAATG. After subcloning into pENTR-D-TOPO (Invitrogen, Carlsbad, USA) the coding sequence was transferred to the E. coli expression vector pDEST17 (Invitrogen, Carlsbad, USA) For protein expression the plasmids was transformed into the yeast (SynAas) strain INVSc1 from Invitrogen or E. coli (ACP) expression strain BLR(DE3)pLys (Merck, Darmstadt, Germany). Protein expression was induced through addition of 2% galactose (yeast) or 1mM IPTG (E. coli) respectively. Protein purification carried out using Ni-NTA-agarose (Qiagen) according to the manufacturer’s instructions with modifications. Concentrations and purity of the proteins were confirmed by SDS-PAGE (Supplemental Figure 5).

Liposome assay

Liposomes were prepared as described previously (Takei et al., 1998) with some modifications. 5 mg phosphatidylcholine were dissolved in chloroform, dried under nitrogen and rehydrated 0.3 M sucrose solution for 1 h. The formed liposomes were suspended in 25 mM Tris-HCl buffer pH 8 and standardized to 200 nm size using lipid extruder (Avanti Polar Lipids, Alabama, USA). Liposomes were pelleted at 160,000 g in a Beckmann ultracentrifuge. The liposomes were incubated with 500 µl Tris HCl pH 8 containing 2µM of [1-14C]-linolenic acid (Hartmann Analytic) for 10 min at 30°C followed by three wash steps with Tris-HCl pH 8. The [1-14C]-linolenic acid loaded liposomes were incubated with 400 µl of acyl-ACP-synthetase assay according to Kaczmarzyk et al. (2010) for 15 min at 30°C. Liposomes and supernatant were separated by centrifugation at 16,000 g counted separately in a Beckmann scintillation counter.
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Figure Legends:

Figure 1:  

A: α-linolenic acid-resistant growth of Δsynaas.  
Growth assays of *Synechocystis* wild type and Δsynaas suspension cultures dilution series pre-grown in control solution (10⁰ = OD₇₅₀ 0.05). 5 µl of suspension culture were spotted on plates supplemented with α-linolenic acid as indicated and incubated at constant light for 7 d.

B: Aliquots of *Synechocystis* wild-type and Δsynaas liquid culture adjusted to OD₇₅₀ 0.25, supplemented with α-linolenic acid as indicated and incubated at constant light for 24 h.

C: Growth curves of wild-type and mutant cells in the presence or absence of 40 µM α-linolenic acid. Suspension cultures were adjusted to an OD₇₅₀ of 0.25 and OD₇₅₀ monitored for 48 hr in the absence (left panel) or presence (right panel) of α-linolenic acid.

Figure 2: Toxic effect of α-linolenic acid on photosynthesis.  
The photosynthetic electron transport rate (ETR) of *Synechocystis* wild-type and Δsynaas cells after different periods of incubation with 150 µM α-linolenic acid (open symbols) or in control conditions (closed symbols). Error bars: SEM, n = 3.

Figure 3: Impact of α-linolenic acid on *Synechocystis* cell structure.  
Electron micrographs of individual wild-type and Δsynaas cells after different periods of α-linolenic acid incubation (0 h, 6 h, 10 h). Wild-type cells accumulate dark, lipophilic substances with increasing incubation time (white asterisks at time point 10 h) and display disintegration of thylakoid membranes and subcellular structure in general. Th: thylakoid membrane, Cb: carboxysome, DNA: nucleosome, Cy: cytoplasm. Scale bar = 0.5 µm.

Figure 4: Accumulation of α-linolenic acid in *Synechocystis* cells.  
A: Short term accumulation of [¹⁴C]-labeled α-linolenic acid (33 µM) in wild-type and Δsynaas cells.

B: Long term incubation of α-linolenic acid: Wild-type and Δsynaas cells were incubated with 150 µM α-linolenic acid.
Circles and asterisks indicate significant (p ≤ 0.01) differences compared to wild type at the same time point or compared to time point zero, respectively. Error bars: SEM, n =3.

C: Fatty acid profile of total lipids in wild type and Δsynaas upon long-term incubation with α-linolenic acid (18:3(n-3)). Only the 18:3(n-3) concentration in wild-type cells is substantially increasing over time and other fatty acids remain unaltered.

Figure 5: Thin layer chromatography of total lipids. Total lipids were extracted from wild-type and Δsynaas cells after 0, 6 and 10 h of α-linolenic acid incubation. A clear increase in free α-linolenic acid (FFA), monogalactosyl diacylglycerol (MGDG), sulfoquinovosyl diacylglycerol (SQDG) and phosphatidylglycerol (PG) can be observed in wild-type cells.

Figure 6: Hypersensitivity phenotype of Δslr1736 and phenotype rescue in the Δslr1736/Δsynaas double mutant. Five microliter (10^0 = OD_{750} 0.05) of wild type, Δslr1736, Δslr1736/Δsynaas and Δsynaas suspension cultures pre-grown in low light conditions (15 µE m^{-2} s^{-1}) were spotted on plates supplemented with α-linolenic acid as indicated and incubated at high-light conditions (300 µE • m^{-2} s^{-1}) for 7 d.

Figure 7: Increased sensitivity to α-linolenic acid by overexpression of SynAas in yeast.

A: wild type, wild type/SynAas, Δfat1, Δfat1/SynAas cultures were diluted to OD_{600} 0.05 and grown in liquid YNB media supplemented with 5.5 mM glucose, 1% tergitol and 3.6 mM α-linolenic acid. EtOH was used as control. Asterisks indicate significantly (p ≤ 0.01) different values compared to wild type. Error bars: SEM, n = 4-5

B: wild-type, wild-type/SynAas, Δfat1, Δfat1/SynAas cells were diluted to OD_{600} 0.05 and spotted on YNB plates supplemented with 0.55 mM glucose, 1% tergitol and 3.6 mM α-linolenic acid. EtOH was used as control.

Figure 8: Free fatty acids in membranes can serve as substrate for SynAas.
Liposomes loaded with $\alpha$-$^{14}$C]linolenic acid were incubated for 15 min with 10 µg of purified SynAas, 15 µM ACP and co-factors. Liposomes and supernatant were separated by ultracentrifugation and radioactivity in supernatant and liposomal fraction was measured. An acyl-ACP concentration of 10 nmol $\cdot$ l$^{-1}$ in the assay supernatant (A) corresponds to an amount of 1 pmol $\alpha$-linolenoyl-ACP produced per µg SynAas. Different letters indicate significantly different values ($p \leq 0.01$). Error bars: SEM, $n = 6$.

Figure 9: Model for SynAas-mediated fatty acid membrane translocation in *Synechocystis* cells.

Free fatty acids like $\alpha$-linolenic acid integrate into the plasma membrane and are retrieved from the membrane phase by SynAas action through simultaneous activation to acyl-ACP (large inset). *Synechocystis* wild-type cells accumulate an excess of $\alpha$-linolenic acid in cell lipids when exposed to elevated concentrations in the external media that leads to lipid peroxidation. In regular growth conditions lipid peroxidation can largely be prevented through the presence of $\alpha$-tocopherol. LD: lipophilic droplets (as depicted in Fig. 3); ACP: acyl carrier protein.
