Rapid Induction of Lipid Droplets in \textit{Chlamydomonas reinhardtii} and \textit{Chlorella vulgaris} by Brefeldin A

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Abstract

Algal lipids are the focus of intensive research because they are potential sources of biodiesel. However, most algae produce neutral lipids only under stress conditions. Here, we report that treatment with Brefeldin A (BFA), a chemical inducer of ER stress, rapidly triggers lipid droplet (LD) formation in two different microalgal species, \textit{Chlamydomonas reinhardtii} and \textit{Chlorella vulgaris}. LD staining using Nile red revealed that BFA-treated algal cells exhibited many more fluorescent bodies than control cells. Lipid analyses based on thin layer chromatography and gas chromatography revealed that the additional lipids formed upon BFA treatment were mainly triacylglycerols (TAGs). The increase in TAG accumulation was accompanied by a decrease in the betaine lipid diacylglycerol N,N,N-trimethylhomoserine (DGTS), a major component of the extraplastidic membrane lipids in \textit{Chlamydomonas}, suggesting that at least some of the TAGs were assembled from the degradation products of membrane lipids. Interestingly, BFA induced TAG accumulation in the \textit{Chlamydomonas} cells regardless of the presence or absence of an acetate or nitrogen source in the medium. This effect of BFA in \textit{Chlamydomonas} cells seems to be due to BFA-induced ER stress, as supported by the induction of three homologs of ER stress marker genes by the drug. Together, these results suggest that ER stress rapidly triggers TAG accumulation in two green microalgae, \textit{C. reinhardtii} and \textit{C. vulgaris}. A further investigation of the link between ER stress and TAG synthesis may yield an efficient means of producing biofuel from algae.

Introduction

Biofuel production from food crops is steadily increasing, resulting in competition for agricultural land between food and fuel production. To retain food sources for an ever-increasing population, it is imperative that new feedstocks for biofuel be identified that can replace fossil fuel [1]. Many microalgae grow rapidly and produce a large amount of biomass, and under certain cultivation conditions, can also store large amounts of oil that can readily be converted into biodiesel [2]. Microalgae have thus been suggested as an alternative source for green renewable energy production [3].

\textit{Chlamydomonas reinhardtii} is a model organism for studying many biological processes, including photosynthesis and flagella function [4]. More recently, it was also used to study microalgal lipid metabolism. This photosynthetic green alga uses sunlight and CO$_2$ to produce chemical energy in the form of carbohydrates and lipids, and requires nitrogen to synthesize the proteins necessary for cell growth [4]. Under stress conditions (notably nitrogen depletion), \textit{Chlamydomonas} starts to accumulate significant amounts of neutral lipids (mainly triacylglycerols, TAGs) in distinct cellular organelles called lipid droplets (LDs) [5–7]. The fatty acids required for TAG assembly are either synthesized \textit{de novo} or produced from recycled acyl chains from membrane lipids [5–7]. Under culture conditions widely used in laboratories, exogenously supplied acetate boosts TAG production \textit{via the de novo} pathway. The exact contribution of each pathway to TAG synthesis under specific conditions remains to be elucidated.

Among the many stress conditions, nitrogen starvation is the most effective means of triggering oil accumulation in algae [7]. However, this approach is not ideal because i) nitrogen depletion reduces photosynthesis and thus the overall production of biomass and ii) at the industrial level, nitrogen removal is energy intensive, time-consuming, and costly [8]. Thus, it would be beneficial to identify methods of inducing oil accumulation in cells that do not involve nitrogen starvation and that can be used to culture many different algae [2].

Brefeldin A (BFA) is a drug well-known for its inhibitory effect on vesicular transport from the endoplasmic reticulum (ER) to the Golgi, which causes retrograde transport of vesicles from the Golgi
to the ER [9]. BFA thus disrupts trafficking of newly synthesized and modified proteins and membrane lipids (mainly phospholipids and sterols) to their final destinations, causing unfolded protein accumulation and lipid composition changes [10,11], all of which are symptoms of ER stress. Notably, the ER stress induced by BFA treatment induces neutral lipid accumulation in Saccharomyces cerevisiae, with corresponding increases in the number of cellular LDs [11,12]. ER stress can be detected in organisms by monitoring the expression levels of ER stress marker genes, including BiP (lumenal binding protein) and SAR1 (secretion associated membrane protein 1).

Here, we report that BFA treatment rapidly induces LD formation in two species of microalgae, Chlamydomonas reinhardtii and Chlorella vulgaris, and that the lipids induced by BFA treatment are mainly TAGs. Our chemical analysis of lipids suggests that this neutral lipid accumulation is due to perturbation of lipid homeostasis at the ER and other extra-plastidial membranes. In support of this explanation, BFA induces the expression of several ER stress marker gene homologs in Chlamydomonas reinhardtii, suggesting that ER stress causes TAG accumulation. Interestingly, the lipid induction by BFA is very fast, and occurs regardless of whether or not nitrogen or acetate is present in the medium, suggesting that the drug induces LD accumulation via a pathway independent of that triggered by nutrient starvation.

Materials and Methods

Cell Culture

The Chlamydomonas reinhardtii strains CC-503 wild type mt+ (ca92) and CC-125 wild type mt+ (137c) were obtained from the Chlamydomonas Genetics Center (USA). Chlamydomonas strains were cultured in Tris acetate phosphate (TAP) medium [12] at 25°C under continuous light with shaking. Chlorella vulgaris cells were obtained from the Biological Resource Center (BRC, Korea) and cultured in BG11 medium [13] under the same conditions as used for Chlamydomonas.

LD Induction by BFA Treatment

Brefeldin A (LC Labs) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 50 mg mL⁻¹ and stored at −20°C until use. An aliquot of this stock solution was added to cell culture media to obtain working concentrations. The same volume of DMSO was added to solvent control samples. In support of this explanation, BFA induces the expression of several ER stress marker gene homologs in Chlamydomonas reinhardtii, suggesting that ER stress causes TAG accumulation. Interestingly, lipid induction by BFA is very fast, and occurs regardless of whether or not nitrogen or acetate is present in the medium, suggesting that the drug induces LD accumulation via a pathway independent of that triggered by nutrient starvation.

LD Staining and Observation by Fluorescence Microscopy

LDs in Chlamydomonas and Chlorella cells were stained with Nile red (Sigma) [14]. The cells were incubated with Nile red at a final concentration of 1 μg mL⁻¹ (prepared from a stock solution of 0.1 mg mL⁻¹ in acetone) for 30 min in the dark at room temperature (~25°C) [7]. Stained cells were then observed under fluorescence microscopy (Nikon, Optihot-2, Japan) using optical filters designed for fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC).

Quantification of LDs by Fluorescence Spectrophotometry and Flow Cytometry

To further quantify the fluorescence signal, Nile red intensity was measured using a Safire fluorescence spectrophotometer (TECAN, Switzerland) with a 488-nm excitation filter and a 565-nm emission filter. Flow cytometry of Chlamydomonas was performed on a FACS Calibur Flow Cytometer (Becton Dickinson). A 10 μL aliquot of 100 μg mL⁻¹ Nile red in acetone was added to 5.0×10⁶ cells in 1 mL of TAP medium 30 min before flow cytometry analysis. Twenty thousand cells were analyzed without gating. The red fluorescence of Nile red was plotted on a histogram.

Measurement of Cell Concentration

Cell growth was monitored by measuring the optical density (OD) at 750 nm using a Safire fluorescence spectrophotometer (TECAN). The absorbance of 750-nm light usually correlates well with the biomass of the culture [15]. Cell number was also counted using a hemocytometer.

Lipid Extraction and Quantification

Total lipids were analyzed as described in The Plant Organelle Database 2 [16] [Kuo Nishida, Lipid extraction from Arabidopsis mitochondria, http://podb.nibb.ac.jp/Organelome/bin/findFunctional?status=confirm&method=1&Species=&Keywords=&Category=Biological assays]. CC-503 cells (approximately 5.0×10⁶) were grown in TAP medium containing either BFA or DMSO alone (solvent control) and subjected to lipid extraction. Briefly, cells were harvested by centrifugation at 2,000 g for 20 min. The pellets were immersed in 2 mL of boiling isopropanol and heated for 5 min at 80°C to inactivate lipases. After cooling, 2 mL each of methanol and chloroform and 1.24 mL of water were added to the sample and the mixture was vortexed for 5 min. The extract was centrifuged (10 min, 2,000 g), the resultant supernatant decanted to a new 10-mL screw-capped glass tube, and the pellet re-extracted with 2 mL of methanol, 1 mL of chloroform, and 0.8 mL of water by vortexing. After centrifugation (10 min, 2,000 g), the supernatant was recovered by decantation, combined with the first supernatant, and then washed with 3 mL of 0.9% KCl (w/v) by vigorous shaking. After centrifugation (10 min, 2,000 g), the lower layer was recovered and the solvent was evaporated under nitrogen stream. To quantify TAGs, dried lipid residues were dissolved in chloroform and separated on silica gel TLC using a solvent mixture that facilitates the separation of neutral lipids [80/30/1 (v/v/v), hexane/diethylthylacetate]. For separation of phospholipids and polar acyl lipids, two-dimensional TLC was performed [first dimension: 160/60/40/20 (v/v/v/v) acetone/toluene/methanol/water; second dimension, 170/25/25/4 (v/v/v/v) chloroform/methanol/acetate/water]. Lipid spots were visualized under UV after spraying with 0.01% (w/v) primuline (Sigma) dissolved in acetone: H₂O (4:1 v/v). Corresponding lipid bands were recovered from the plate and quantified by gas chromatography (GC) (SHIMADZU GC-2010, HP-INOWAX capillary column, 30 m, 0.25 mm) after being converted to fatty acid methyl esters (FAMEs) via an acid-catalyzed transesterification protocol previously described by Li et al. 2006.

RNA Extraction and Quantification

Total RNA was extracted according to the phenol/chloroform method described in the [17], with a few modifications. Total RNA was isolated using RNA extraction buffer (250 mM Tris HCl [pH 9.0], 250 mM NaCl, 50 mM EDTA, 345 mM p-aminosaliclylic acid, 27 mM trisopropyl naphthlene sulfonic acid, 250 mM β-mercaptoethanol, 0.024% [v/v] phenol) [18], and cDNA was synthesized by reverse transcription using 4 μg of total RNA. Real-time PCR was performed using primer sets designed to amplify Chlamydomonas BiP homologs CrBiP1 (5’-AGT GAG CCC TTC TTT TAG AAG CT-3’) and 5’-TCT CCA TGG ATG TAG GCC TTT AAG CTA-3’; CrBiP2 (5’-TAT CCG TAG TGC ATT TGT TGG AA-3’) and 5’-GTT GAA GGA AGC.
AGA ACA AAA GA-3'), or CsHR1 (5'-CGA GGA GAT TCA ATT GGG CG-3' and 3'-CGG TGG GAA TGT CGA TCT TG-3'), according to the information in Phytozome v9.1 at www.phytozome.net. The real-time PCR results were normalized by the level of RPL17 expression [19].

Chlorophyll Measurements
Chlorophyll content was measured using the ethanol extraction method [20]. A 1-mL aliquot of culture, at a concentration of 5.0 x 10^6 cells mL^-1, was centrifuged, and the pellet was resuspended in 95% ethanol. Cellular debris was removed by centrifugation and chlorophyll a and b levels in the supernatant were determined by measuring optical absorbance at 648 nm and 664 nm, respectively. Total chlorophyll content was calculated as described previously [20].

Results

BFA Treatment Induces LD Formation in the Model Microalga Chlamydomonas reinhardtii

In an effort to identify alternative methods of triggering oil accumulation in green microalgae, we evaluated the effects of a well-known vesicle trafficking inhibitor, brefeldin A (BFA), on lipid droplet (LD) formation in Chlamydomonas reinhardtii. As an initial test, we incubated a culture of cell wall-less Chlamydomonas strain C-503 in TAP medium until the mid-log phase (approximately 5.0 x 10^6 cells mL^-1), and then treated the cells with BFA for 4 h at a final concentration of 75 μg mL^-1. We monitored the occurrence of LDs in BFA-treated cells by staining the samples with a fluorescent indicator dye for lipid bodies, Nile red, and then monitoring them by fluorescence microscopy.

Whereas numerous fluorescent LDs were observed in BFA-treated cells, none or only a few were present in control cells (treated only with DMSO, the solvent used for dissolving BFA) (Fig. 1A). LDs were highly fluorescent under either the TRITC or FITC filter set, but they were more clearly distinguishable from the large chloroplast when the FITC filter set was used. Time dependency tests using a TECAN fluorescence spectrophotometer revealed that, in normal TAP medium supplemented with nitrogen (+N) and acetate (+Ac), the addition of BFA rapidly (within 2 h) induced the formation of LDs, as gauged by an increase in Nile red fluorescence (Fig. 1B). The Nile red fluorescence signal plateaued between 8 and 15 h after BFA addition. However, BFA treatment for 8 h did not cause any significant change in total chlorophyll content or chlorophyll a/b ratios (Fig. S1). Incubation beyond 15 h did not increase the Nile red fluorescence signal, but decreased the reading at OD 750, a parameter indicative of cell biomass (Fig. 1C), suggesting damage to the cells and a consequent decline in biomass. Thus, for the following experiments, we treated the cells with BFA only for an 8-h period, unless otherwise specified.

To examine the effect of BFA at the cell population level, we used another strain of Chlamydomonas reinhardtii, CC-125, which has a cell wall. To allow BFA to penetrate the cell wall efficiently, the cells were treated with BFA for 4 h, then stained with Nile red. As shown in figure S2, BFA induced LD formation, and the extent of LD formation correlated well with the length of time of sonication, suggesting that the more BFA that enters the cells, the more LDs the cells accumulate.

Dose-dependent and Growth Phase-dependent Effects of BFA Treatment

To decipher the mechanisms underlying BFA action, Chlamydomonas cells were treated with different concentrations of BFA, ranging from 0 to 75 μg mL^-1. As shown in figure 2A, levels of Nile red fluorescence were similar at 2 h, but after 8 h, higher levels of Nile red fluorescence were detected in cells treated with higher concentrations of BFA (Fig. 2A). We then tested the effect of BFA treatment on the induction of lipid bodies at different growth phases of the Chlamydomonas cell culture. BFA treatment increased Nile red fluorescence at all growth phases, but the drug was most effective when applied at the early log phase (48 h) (Fig. 2B).

BFA Treatment Induces TAG Accumulation at the Expense of DGTS

In Chlamydomonas reinhardtii, LDs formed upon nitrogen starvation contain mainly triacylglycerols (TAGs) [21]. To examine if the LDs induced by BFA treatment also contain mainly TAGs, total lipids were extracted from CC-503 cells after an 8-h treatment with BFA. Analysis of a TLC plate stained with primuline and viewed under UV illumination revealed that more TAGs accumulated in BFA-treated cells than in control cells (Fig. 3A). Recovery of the bands corresponding to the TAGs and quantification by GC confirmed that the concentration of TAGs was 34% greater in the treated cells compared to the control (Fig. 3B).

BFA treatment significantly increased the levels of 16:0, 16:1(7), 18:0, 18:1(9), 18:1(11), 18:2(9,12), and 18:3(5,9,12) and significantly decreased the levels of 16:4(4,7,10,13) and 18:3(9,12,15) in TAGs (Fig. 3C). Especially noteworthy were the increase in 18:3(5,9,12), the major fatty acid of diacylglycerol-V, V, V-trimethylhomoserine (DGTS), a non-plastidial lipid [22–25], and the decrease in 16:4(7,10,13) and 18:3(9,12,15), the two major components of plastidial lipids [25]. These fatty acid profiles indicated that the fatty acid substrates used for BFA-induced TAG assembly were derived mainly from non-plastidial membrane lipids.

Changes in Lipid Composition Induced by BFA Treatment

To further identify which membrane lipids were converted into TAGs in BFA-treated cells, polar lipid compositions were examined as described in Materials and methods. Total lipids were extracted from cells and major polar lipids were separated by two-dimensional TLC and quantified by GC after derivatization into fatty acid methyl esters.

The amount of total lipids did not differ between BFA-treated and control cells (Fig. 3D), despite a small increase in TAG accumulation in BFA-treated cells (Fig. 3B). TAG content was much lower than that of total membrane lipids (compare the y-axes of Figs. 3C and 3D). DGTS was reduced by over 30% upon BFA treatment, whereas a moderate increase in phosphatidylethanolamine (PE) and a moderate decrease in digalactosyldiacylglycerol (DGDG) were observed (Fig. 3D). The content of other lipids changed much less. The reduction in DGTS by BFA further supports the hypothesis that a portion of recycled acyl chains from the non-plastidial membrane lipids was used for TAG synthesis in BFA-treated cells.

Brefeldin A Induces Lipid Bodies in Chlamydomonas
Figure 1. BFA treatment induces lipid droplet (LD) formation in *Chlamydomonas reinhardtii strain CC-503*. (A) Images of Nile red-stained LDs in BFA-treated cells. Images were acquired after cells were incubated for 4 h with BFA at a concentration of 75 µg mL⁻¹. DMSO was used as a solvent control (Con). Bar = 10 µm. Nile red fluorescence was viewed using either the 465–495 nm excitation/515–555 nm emission channel (FITC, green) or the 540/25 nm excitation/605/55 nm emission channel (TRITC, red). (B) Time-dependent effect of BFA treatment on the fluorescence intensity of LDs stained with Nile red. The fluorescence was quantified using a fluorescence spectrophotometer with a 488-nm excitation filter and a 565-nm emission filter. AU = arbitrary units. (C) Growth of the Chlamydomonas culture treated with BFA, measured by reading at OD750. (D) Quantification of Nile red fluorescence in individual BFA-treated cells by flow cytometry. We examined 20,000 cells subjected to two different conditions (DMSO solvent control, and BFA at 75 µg mL⁻¹). AU = arbitrary units.

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BFA can Induce TAG Synthesis via Pathways that are Different from Acetate Boosting or Nitrogen Starvation

We then tested whether BFA treatment of *Chlamydomonas* induces TAG synthesis even in the absence of acetate or nitrogen sources in the medium. *Chlamydomonas* CC-503 cells grown in normal TAP (+N, +Ac) medium were collected by centrifugation and resuspended in new TAP medium with or without acetate. After an 8-h treatment with BFA, cells were stained with Nile red and the LD fluorescence was quantified using a TECAN fluorescence spectrophotometer. BFA treatment increased the LD content of cells grown in TAP media either with or without acetate (Fig. 4A). However, BFA induced LD more effectively in the presence of acetate than in its absence: compared to the solvent control, LD fluorescence increased by 40% in the presence of acetate, and by 25% in the absence of acetate.

To determine whether BFA-induced oil accumulation depends on the presence of nitrogen, *CC-503* cells grown in nitrogen-deficient TAP medium for 1 day were treated with BFA for 8 h, and the LD fluorescence was quantified by fluorescence spectrophotometry. Nitrogen depletion induced an increase in fluorescence (Fig. 4B), and BFA treatment further increased LD fluorescence (Fig. 4B). To obtain biochemical evidence of this, total lipids were extracted from *CC-503* cells grown for 2 days under nitrogen starvation conditions and then treated with BFA. Since cells were weak under these growth conditions, BFA treatment lasted only for 3 h in this experiment. TLC and GC analyses revealed that the total TAG content was 19% higher in BFA-treated cells than in control (DMSO-treated) cells under nitrogen starvation (Fig. 4C). However, in contrast to the changes in fatty acid composition induced by BFA under nitrogen-
Figure 3. Changes in lipid composition of BFA-treated *Chlamydomonas reinhardtii CC-503*. Cells were grown to the mid-log phase, treated with BFA (75 µg mL⁻¹) or DMSO (solvent control, Con) for 8 h, and then lipids were analyzed. (A) Accumulation of TAGs on a TLC plate as revealed by staining with primuline. The bands in the box are TAGs with the same Rf value as soybean TAGs. (B) BFA-treated cells accumulate higher amounts of TAGs. (C) BFA-treated cells accumulate higher amounts of fatty acids. (D) BFA-treated cells accumulate higher amounts of membrane lipids.
The fatty acid composition of TAGs isolated from BFA-treated and control (Con) cells. In (B) and (C), three replicates were averaged, and the SEs are shown. Significant differences, as determined by Student’s t-test, are indicated by asterisks (*P<0.05, **P<0.01). (D) Comparison of the major lipid classes between BFA-treated and control (Con) cells. Averages from two replicate experiments and their standard deviations are shown. PI, phosphatidylinositol; PE, phosphatidylethanolamine; PG, phosphoglyceride; DGTs, diacylglyceroltrimethylhomoserine; SQDG, sulfoquinovosyl-diacylglycerol; MGDG, monogalactosyldiacylglycerol; and DGDG, digalactosyldiacylglycerol.

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Figure 4. BFA-induced LD formation is independent of the presence of an acetate or nitrogen source in the medium. (A) Nile red fluorescence from CC-503 cells grown in normal TAP (+N, +Ac) medium, transferred to medium with or without acetate, and incubated in continuous light. After an 8-h incubation, Nile red fluorescence was measured for control (Con: DMSO solvent control) and BFA-treated cells using a fluorescence spectrophotometer equipped with a 488-nm excitation filter and a 565-nm emission filter. AU = arbitrary units. (B) Nile red fluorescence of CC-503 cells grown in nitrogen-replete or nitrogen-deficient medium for 1 day, and then treated with BFA for 8 h. AU = arbitrary units. (C) Total TAGs from control (Con) or BFA-treated cells starved in nitrogen-deficient medium for 2 days. DMSO and BFA treatment lasted for 3 h. In (b) and (c), three replicates were averaged, and the SEs are shown. Significant differences, as determined by Student’s t-test, are indicated by asterisks (*P<0.05, **P<0.01). (D) Fatty acid composition of the TAGs extracted from the solvent control (Con) and BFA-treated cells. Both samples had been starved of nitrogen for one day. Averages from three replicate experiments are shown. No statistically significant differences were found.

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BFA Induces ER Stress Marker Gene Expression in *Chlamydomonas*

To test whether BFA induces ER stress in *Chlamydomonas*, we first searched the *Chlamydomonas* genome for homologs of ER stress marker genes. When the amino acid sequence of AtBiP1, AtBiP2, or AtBiP3 was used in the search, we found that *Chlamydomonas* has two genes with high similarity to AtBiP proteins, and we named these genes *CrBiP1* (g1475) and *CrBiP2* (Cre02.g080600). *CrBiP1* and *CrBiP2* were 69–71% similar in amino acid sequence to AtBiP1, AtBip2, and AtBiP3 (Fig. S3). A search using the amino acid sequence of AtSAR1 (At1g09180) revealed one similar gene, which we named *CrSAR1* (Cre11.g468300). *CrSAR1* shared 82% amino acid sequence similarity with AtSAR1 (Fig. S4). We then tested whether these genes were induced in *Chlamydomonas* by dithiothreitol (DTT), which has been reported to induce ER stress in many organisms, including *Arabidopsis*, rice, and yeast [26–28]. Treatment with 6 mM DTT for 2 h induced the expression of *CrBiP1*, *CrBiP2*, and *CrSAR1* (Fig. 5A), suggesting that these genes can be used as ER stress marker genes in *Chlamydomonas*, as their homologous genes are used in other organisms [26,29,30]. Treatment of the *Chlamydomonas* cells with 75 μg mL⁻¹ BFA for 8 h caused a dramatic increase in the expression of *CrBiP1*, *CrBiP2*, and *CrSAR1* (Fig. 5B).

BFA Treatment also Induces LD Formation in the Industrially Profitable Alga *Chlorella vulgaris*

To determine whether our method of LD induction could be applied to industrially profitable algae, we tested the effect of BFA addition on *Chlorella vulgaris*, an industrially cultivated microalg [31]. BFA treatment for 4 h did not significantly alter cell growth (Fig. 6A, right), but effectively induced the formation of LDs (Fig. 6A left, B). Furthermore, the effect was even stronger in cells sonicated for 30 s before BFA treatment (Fig. 6A), presumably because the sonication facilitated drug delivery into the cells.

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**Figure 5.** Expression levels of ER stress marker genes in BFA- and DTT-treated *Chlamydomonas reinhardtii CC-503*. Cells in mid-log phase culture were treated with 6 mM DTT dissolved in TAP solution for 2 h, or with BFA (75 μg mL⁻¹) dissolved in DMSO for 8 h. Control cells were treated with TAP solution or DMSO, respectively. Transcript levels of BiP homologs (g1475 and Cre02.g080600) and a SAR1 homolog (Cre11.g468300) were analyzed, and fold changes compared with the expression level in the control samples are presented. The averages and standard errors from two independent experiments are shown. Significant differences, as determined by Student’s t-test, are indicated by asterisks (*P < 0.05, **P < 0.01). doi:10.1371/journal.pone.0081978.g005
In the present study, we report that BFA rapidly induces LD formation in two different algae, *Chlamydomonas reinhardtii* (Fig. 1, Fig. S2) and *Chlorella vulgaris* (Fig. 6). The response was greatest during the early log phase of culture (Fig. 2B), and analysis of individual lipid classes revealed that DGTS content decreased, but without altering the total amount of phospholipids and galactolipids (Fig. 3D), suggesting that acyl recycling and assembly into TAGs increased upon BFA treatment. This BFA effect was observed regardless of whether an acetate or nitrogen source was present in the medium or not (Fig. 4).

BFA commonly induces ER stress in many different organisms. We suggest that BFA induces ER stress in *Chlamydomonas* too, without altering the total amount of phospholipids and galactolipids (Fig. 3D), suggesting that acyl recycling and assembly into TAGs increased upon BFA treatment. This BFA effect was observed regardless of whether an acetate or nitrogen source was present in the medium or not (Fig. 4).

**Figure 6. BFA treatment induces LD formation in Chlorella vulgaris.** (A) A 4 h BFA (75 μg/ml) treatment increases Nile red fluorescence (left) without altering cell density (right). Nile red fluorescence resulting from LDs was quantified using a fluorescence spectrophotometer. AU = arbitrary units. Cells represented by the right-most two bars of each panel were sonicated for 30 s at the 40 watt (W) setting of the sonicator (Vibra Cell™, VC 130PB). Averages from three replicate experiments, and their standard errors are shown. (B) Images of *Chlorella* cells treated with 75 μg mL⁻¹ BFA and stained with Nile red. Bar = 10 μm. DMSO was used as a solvent control (Con).

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because BFA treatment dramatically increased the expression of ER stress marker homologs, CrBiP1, CrBiP2, and CrSAR1 (Fig. 5). Supporting this possibility, DTT, a drug known to induce ER stress in many different organisms [26,30,32], also induced the expression of the three genes (Fig. 5). BFA treatment inhibits membrane trafficking from the ER to Golgi membranes, thereby promoting retrograde vesicle transport from Golgi membranes to the ER. This situation might provide ample substrates for the assembly of TAGs, causing LD accumulation in BFA-treated cells. In accordance with this hypothesis, the concentration of DGTS, a lipid synthesized and exported from the ER, was decreased upon BFA treatment (Fig. 3D). Thus, we suggest that DGTS, which is retrieved from other membranes into the ER, is recycled and provides the fatty acid source for the assembly of TAGs in BFA-treated cells. This explanation is further supported by the high level of 18:3(5,9,12) in the TAGs of BFA-treated cells compared to those of control cells (Fig. 3C). The 18:3(5,9,12) fatty acid is the major fatty acid present in DGTS, and is rarely found in chloroplastic lipids [25]. However, the increase in TAG levels is much smaller than the decrease in DGTS, and the content of several other lipids was also altered. Thus, BFA seems to cause a serious perturbation in lipid homeostasis, and a part of the degradation product of membrane lipids is converted into TAGs. To accurately determine the sources of fatty acids for BFA-induced TAG synthesis, pulse-chase experiments should be conducted in the future.

To perceive and overcome the ER stress, animal cells invoke pathways that increase the protein folding capacity, decrease the protein production at the ER, and promote lipogenesis [26,27]. Similar ER stress responses are observed in some plant cells. ER stress activates many lipid metabolic enzymes in maize and soybean cells, and increases TAG accumulation in maize endosperm [29]. Our experiments revealed BFA-induced TAG synthesis in two species of green algae, suggesting that similar alterations in lipid metabolism occur in response to ER stress in green algae. Consistent with this possibility, a homolog of inositol-requiring enzyme 1 (IRE1), a protein kinase/ribonuclease with a central role in the ER stress response, exists in the Chlamydomonas genome (g0693 in Phytozome v9.1 at www.phytozome.net). IRE1 is found in yeast, nematodes, fruit flies, plants, and animals, and is thus considered to function in the most ancient pathway of the ER stress response [33]. Further studies of ER stress responses in algae might provide important insight into the evolution of the ER stress response.

Figure 7. A hypothetical model explaining BFA-induced LD formation.
1. BFA inhibits the protein trafficking from the ER to Golgi
2. Retrograde transport & Golgi-ER aggregation
3. Accumulation of immature proteins and disruption of sterol homeostasis
4. Change in lipid synthesis
   DGTS \( \rightarrow \) TAG
5. LD formation
   Sequestration of immature proteins

What might be the functions of LDs in ER stress? Fungi [11,34] and animals [35] were previously found to accumulate LDs in the presence of ER stress. LD formation may be a resistance mechanism against BFA toxicity, which is characterized by the
Previous approaches to increase TAG content in *Chlamydomonas* focused on increasing the activity of enzymes involved in TAG biosynthesis [40], or disrupting starch synthesis [3]. However, both strategies depend on nitrogen starvation or other stress conditions that require long-term treatment and are expensive. Here, we demonstrated that BFA rapidly induces LD formation in two microalgal species, *Chlamydomonas* and *Chlorella*. It is particularly interesting that the drug was effective regardless of whether or not an exogenous acetate or nitrogen source was present in the growth medium (Fig. 4). Therefore, the drug can be used to convert membrane lipids into TAGs after harvest of biomass grown under optimal conditions. This is valuable, since neutral lipids such as TAGs are much easier to convert into fuel than membrane lipids. However, the problem in this scheme is that the drug is non-specific and has pleiotropic effects, including protein degradation and, in the case of prolonged treatment, cell death. Thus, it is necessary to pinpoint specifically which aspect of the BFA response alters the lipid content and to use this knowledge to reduce the amount of time needed to produce LDs and increase the portion of neutral lipids harvestable from the algae. Further studies of the detailed mechanisms by which BFA affects lipid metabolism may provide a clue for developing an efficient strategy for microalgae-based biofuel production.

**Supporting Information**

**Figure S1** BFA does not significantly change chlorophyll content. *Chlamydomonas reinhardtii* strain CC-503 cells at the mid-log phase (48 h after the beginning of sub-culture) were treated with 75 µg mL⁻¹ BFA for 8 h, and then chlorophyll content was measured.

**Figure S2** BFA induces LD formation in the **CC-125 line of Chlamydomonas reinhardtii**, which has a cell wall. To facilitate BFA uptake, samples were sonicated for 0, 10, or 30 s before BFA treatment. All images were captured in the FITC channel, except the bottom right image, which was captured in the TRITC channel.

**Figure S3** Multiple sequence alignment of BiP orthologs from Arabidopsis, Chlamydomonas, and Saccharomyces. CLUSTALW (http://www.genome.jp/tools/clustalw) was used for the alignment. Stars indicate conserved amino acids.

**Figure S4** Multiple sequence alignment of SAR1 orthologs from Arabidopsis, Chlamydomonas, and Saccharomyces. CLUSTALW (http://www.genome.jp/tools/clustalw) was used for the alignment. Stars indicate conserved amino acids.

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**Author Contributions**

Conceived and designed the experiments: SK HK IN Y. Li-Beisson Y. Lee. Performed the experiments: SK HK DK YY MO MK-Y TI H-MO. Analyzed the data: SK HK IN Y. Li-Beisson Y. Lee. Contributed reagents/materials/analysis tools: IN H-MO Y. Lee. Wrote the paper: SK HK IN Y. Li-Beisson Y. Lee.
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