C-ferroptosis is an iron-dependent form of regulated cell death in cyanobacteria

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Ferroptosis is an oxidative and iron-dependent form of regulated cell death (RCD) recently described in eukaryotic organisms like animals, plants, and parasites. Here, we report that a similar process takes place in the photosynthetic prokaryote Synechocystis sp. PCC 6803 in response to heat stress. After a heat shock, Synechocystis sp. PCC 6803 cells undergo a cell death pathway that can be suppressed by the canonical ferroptosis inhibitors, CPX, vitamin E, Fer-1, liproxstatin-1, glutathione (GSH), or ascorbic acid (AsA). Moreover, as described for eukaryotic ferroptosis, this pathway is characterized by an early depletion of the antioxidants GSH and AsA, and by lipid peroxidation. These results indicate that all of the hallmarks described for eukaryotic ferroptosis are conserved in photosynthetic prokaryotes and suggest that ferroptosis might be an ancient cell death program.

Introduction

In contrast with accidental cell death, regulated cell death (RCD) relies on a tightly modulated molecular machinery that involves signaling cascades and defined effectors (Galluzzi et al., 2018). In eukaryotes, RCD plays a critical role in essential physiological programs such as embryonic development, differentiation, fertilization, tissue renewal, and immune responses, generally referred to as programmed cell death (PCD; Hakem et al., 1998; Lindsten et al., 2000; Van Hautegem et al., 2015; Yoshida et al., 1998). RCD can also occur when responses to perturbations of the intracellular or extracellular microenvironment fail, as an ultimate attempt to maintain homeostasis (Galluzzi et al., 2016).

Different types of RCD have been described in eukaryotes (Melino et al., 2005; Minina et al., 2013). Among them, ferroptosis was recently reported as an oxidative, iron-dependent form of RCD characterized by the disturbed thiol homeostasis and the accumulation of lipid hydroperoxides to lethal levels. Ferroptosis is present and relevant in animals, plants, and protozoan parasites (Bogacz and Krauth-Siegel, 2018; Conrad et al., 2018; Distéfano et al., 2017; Dixon et al., 2012; Stockwell et al., 2017).

Although less studied and understood, RCD also occurs in prokaryotic microorganisms (Allocati et al., 2015; Bayles, 2014; Dar et al., 2018; Durand et al., 2016).

Cyanobacteria are widely distributed Gram-negative bacteria that are capable of plant-like oxygenic photosynthesis. In particular, cyanobacteria are important components of phytoplankton communities, contributing to a substantial fraction of the global primary production, and are a crucial source of atmospheric oxygen (Whitton, 2012). Despite its ecological and biogeochemical significance, the nature of RCD in cyanobacteria is still not completely understood (Aguilera et al., 2021). Evidence has accumulated on controlled cell death mechanisms triggered in cyanobacteria under unfavorable environmental conditions such as nutrient deprivation, high-light-associated oxidative stress, or osmotic stress, which are indistinctly termed as PCD, apoptotic-like death, or necrotic-like death. Such cell death pathways involve morphological changes, accumulation of reactive oxygen species (ROS), DNA laddering, loss of plasma membrane integrity, and the coordinated participation of redox enzymes, metabolites, and caspase-like proteases (Aguilera et al., 2021; Bidle, 2016; Hu and Rzynski, 2019;
Spungin et al., 2019; Swapnil et al., 2017; Zhou et al., 2020b). RCD has been proposed to optimize differentiation, dynamics, and colony fitness in cyanobacteria (Bar-Zeev et al., 2013; Meeks and Elhai, 2002). In addition, microscopic analyses showed that cyanobacteria symbiotically associated with the water fern Azolla microphylla can follow different RCD pathways with characteristics of metazoan apoptosis, autophagy, necrosis, and autolysis (Zheng et al., 2013).

In this work, we examined whether ferroptosis could be relevant to the model cyanobacterium Synechocystis sp. PCC 6803 (hereafter Synechocystis) under abiotic stresses. We found that Synechocystis follows a cell death program in response to heat stress (50°C) that shows biochemical and morphological features that resemble eukaryotic ferroptosis (Conrad et al., 2018; Distéfano et al., 2017). This cell death is dependent on iron availability and lipid peroxidation, and it is inhibited by canonical ferroptosis inhibitors. Moreover, cell death is characterized by depletion of glutathione (GSH) and ascorbic acid (AsA), and can be prevented by GSH or AsA addition. Since this pathway is also characterized by specific features, we termed this cell death process cyanobacterial-ferroptosis (c-ferroptosis). Altogether, these results suggest that ferroptosis might be an ancient cell death program. In addition, as chloroplasts originated from the endosymbiosis of cyanobacterial-like organisms, the presence of this iron-dependent oxidative cell death pathway in cyanobacteria also points to the evolutionary origin of the chloroplasts role during plant ferroptosis (Distéfano et al., 2017).

**Results**

**Ferroptosis inhibitors prevented cell death triggered by a 50°C-heat stress in cyanobacteria**

To examine whether ferroptosis plays a role during stress-induced cell death in Synechocystis, cells were exposed to different treatments: (1) H2O2, known to promote cell death in a regulated way in the cyanobacterium Microcystis aeruginosa (Ding et al., 2012; Zhou et al., 2020b); (2) 50°C for 10 min (50°C-heat stress [HS]), known to cause cell death in Synechocystis (Suginaka et al., 1999); and (3) 77°C for 10 min, a treatment known to trigger unregulated necrosis in plant roots (Distéfano et al., 2017). Each treatment was applied in the presence or absence of two canonical ferroptosis inhibitors: the lipophilic antioxidant ferrostatin-1 (Fer-1) or the membrane-permeable iron chelator ciclopirox olamine (CPX; Stockwell and Jiang, 2020). Cell death was assessed by three different methodologies following Nomenclature Committee on Cell Death recommendations (Kroemer et al., 2009): drop tests to measure cell survival (Fig. 1 a), fluorescence microscopy using SYTOX Green as a cell death marker (Fig. 1 b and Fig. S1 a), and fluorescein diacetate (FDA) fluorescence quantification by flow cytometry to assess viability (Fig. 1 c). All experiments indicated that cell death triggered by 50°C is significantly prevented by Fer-1 and by CPX (Fig. 1, a–c), suggesting that the 50°C-HS triggers the ferroptotic cell death pathway. In contrast, neither Fer-1 nor CPX was able to prevent cell death triggered by a higher temperature (77°C) or by H2O2. In addition, cell death was significantly prevented when Synechocystis cells were exposed to 50°C-HS but were preincubated with the lipophilic antioxidants liproxstatin-1 (Lipro-1) and vitamin E, which are also recognized as ferroptosis inhibitors (Fig. 1 d). Altogether, these results indicate that the response of Synechocystis cells to 50°C-HS is specific, and can be prevented by canonical ferroptosis inhibitors such as CPX, Fer-1, Lipro-1, and vitamin E (Dixon and Stockwell, 2019; Kagan et al., 2017; Stockwell and Jiang, 2020).

**Role of calcium in the RCD pathway triggered by 50°C in Synechocystis**

To test the effect of calcium on Synechocystis, we exposed cells to 50°C-HS in the presence of CaCl2, EGTA, an extracellular calcium chelator, BAPTA-AM, an intracellular calcium chelator, and a combination of these compounds. 4 h after the treatment, ~70% of 50°C-HS treated cells were found dead (Fig. 2). Surprisingly, when cotreated with CaCl2, only ~10% of the 50°C-HS treated cells died, a value comparable to the control (cells that were not exposed to HS, Fig. 2 a). On the other hand, ~90% of the cells were found dead when treated with EGTA, and less than ~10% were found dead when EGTA and CaCl2 were added together. These results suggest that extracellular calcium can prevent cell death induced by 50°C-HS (Fig. 2 a). However, neither Fer-1 nor CPX was able to prevent cell death in the presence of EGTA (Fig. 2 b).

Cell death triggered by 50°C was significantly prevented by the addition of the intracellular Ca2+ chelator (BAPTA-AM; Fig. 2 a). When 50°C-treated cells were preincubated with CPX and BAPTA-AM, cell death reached ~70%, a value comparable to 50°C-treated cells without inhibitors (Fig. 2 d). This result can be explained by the fact that iron chelators might increase the levels of intracellular calcium, as observed in human cells (Yalcintepe and Halis, 2016). On the other hand, assays combining Fer-1 and BAPTA-AM prevented cell death similarly to the effect of preincubation with BAPTA-AM alone (Fig. 2 d). These results support the role of Ca2+ as a second messenger in this cell death pathway, in which extracellular and intracellular sources might play antagonistic roles.

**The 50°C-HS induces depletion of the antioxidants GSH and AsA**

Ferroptosis is characterized by an early depletion of GSH in animals, and both GSH and AsA in plants (Distéfano et al., 2017; Dixon et al., 2012; Seiler et al., 2008; Skouta et al., 2014). The exposure of Synechocystis cells to 50°C resulted in a decline of GSH and AsA total contents (Fig. 3, a–d). These effects were not prevented by the addition of Fer-1 or CPX, suggesting that GSH and AsA depletion might be an early event in this cell death pathway, as described for plant ferroptosis (Distéfano et al., 2017). The redox status of GSH (percentage of oxidized GSH disulfide [GSSG]) did not show a clear association with the treatments and did not show differences with the addition of Fer-1 or CPX under normal temperature conditions. On the other hand, oxidized AsA content was very low or almost undetectable. Notably, cell death was prevented by preincubation with GSH, AsA, and DTT, suggesting that GSH and AsA depletion is required for HS-induced cell death in Synechocystis (Fig. 3, e and f).
50°C-HS induced rapid accumulation of cytosolic and lipid ROS

In eukaryotic organisms, lipid ROS accumulation plays a central and essential role in ferroptosis. This oxidative burst is prevented by iron chelators (CPX) and lipophilic antioxidants (Fer-1; Bogacz and Krauth-Siegel, 2018; Distefano et al., 2017; Dixon et al., 2012). To investigate if a similar process occurs in Synechocystis, cytosolic ROS and lipid ROS accumulation were measured after inducing cell death by a 50°C-HS. Cytosolic ROS were measured with the probe H2DCFDA, while lipid ROS accumulation was monitored with the probe C11-BODIPY. A maximum of cytosolic ROS was detected 1 h after 50°C-HS (Fig. 4a). This accumulation of cytosolic ROS was prevented by incubation with Fer-1 or CPX (Fig. 4a). The accumulation of lipid peroxides reached a maximum 3 h after the 50°C-HS and was suppressed by both ferroptosis inhibitors (Fig. 4b).

Pre-treatments with polyunsaturated fatty acids (PUFAs) deuterated at bis-allylic positions (D-PUFAs) prevent ferroptosis in both human and plant cells (Yang et al., 2016; Distefano et al., 2017). Likewise, we observed a clear protective effect of D-linoleate (16 h) against the 50°C-HS in Synechocystis (Fig. 4c). Altogether, these results indicate that Synechocystis cells exposed to a 50°C-HS undergo an oxidative, iron-dependent form of cell death remarkably similar to ferroptosis in eukaryotic cells (Bogacz and Krauth-Siegel, 2018; Dangol et al., 2019; Distefano et al., 2017; Dixon et al., 2012).

Redox-lipidomics analysis reveals a lipid oxidation signature of c-ferroptosis

The oxidation of membrane lipids containing PUFAs is one of the main hallmarks that define ferroptosis (Dixon and Stockwell, 2019). To identify the oxygenated lipids that resulted from the
50°C-HS, we performed global redox lipidomics liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis (Kagan et al., 2017; Tyurin et al., 2008; Tyurina et al., 2014). The 50°C-HS induced the accumulation of oxidized species of phosphatidylethanolamine (PE; 36:4-OO and 36:5-OO) and phosphatidylglycerol (PG; 34:2-O, 34:2-OO, 36:3-OO, 34:3-OO, 36:2-O, and 34:3-O). Interestingly, it also induced the accumulation of oxidized species of the sulfo-lipids sulfoquinovosyl diacylglycerols (SQDGs; 34:2-O, 34:2-OO, 34:4-O, 34:4-OO, and 34:3-O), which are a major component of cyanobacterial thylacoids (Nakajima et al., 2018). Remarkably, this accumulation of oxidized species of phospho- and sulfo-lipids was prevented when 50°C-treated cells were preincubated with Fer-1, suggesting that they might act as pro-ferroptotic signals in this system (Fig. 5).

In addition, our results indicated that nonoxidized species of the galactolipid monogalactosyldiacylglycerol (MGDG) accumulated when cells were exposed to 50°C (Fig. S2). These lipids are very abundant in the thylakoid membranes and accumulate in response to environmental stress (Yang et al., 2020).

As this pathway seems to be characterized by particular lipid species peroxidation and calcium signaling, we decided to name it c-ferroptosis.

**Synechocystis shows thylakoid membranes alteration after heat shock**

To gain insight into the morphological changes taking place in our cell death model, *Synechocystis* sp. PCC6803 cells treated with H$_2$O$_2$ or subjected to 50°C or to 77°C were studied using transmission EM (TEM; Fig. 6). The distinctive morphological features of 50°C-treated cells involved the loss of the thylakoids membrane integrity, with evident nonelectron dense zones, and vesiculation (Fig. 6). On the other hand, cells exposed to 77°C showed a
reduction of the cellular volume and electron dense regions, while the thylakoid membranes were not distinguishable (Fig. 6). Cells treated with H2O2 showed cytoplasm vacuolation (Fig. 6).

Caspase-3/7 activities are not induced after the 50°C-HS in *Synechocystis*

In animals, caspases are a well-studied family of evolutionary conserved cysteine-dependent proteases that are implicated in the regulation of several signaling pathways and are considered as the central executioners of apoptosis (Saraste and Pulkki, 2000; Van Opdenbosch and Lamkanfi, 2019). Plants, protists, fungi, and bacteria lack true caspases but contain various homologues (metacaspases and orthocaspases; Klemenčič and Funk, 2018). Even though several of these homologues have been implicated in stress response and cell death in cyanobacteria, their function and regulation are not yet fully understood (Aguilera et al., 2021; Klemenčič et al., 2019). *Synechocystis* sp. PCC 6803 only contains a single orthocaspase (SyOC), which lacks the catalytic dyad. However, although the protein is presumably proteolytically inactive, the gene is highly expressed (Klemenčič et al., 2019).

We monitored caspase-like activity in treated *Synechocystis* cells by using the CellEvent caspase-3/7 green detection reagent, following previous studies on cell death in cyanobacteria (reviewed by Hu and Rzymski, 2019). Caspase-3/7 signals did not increase after exposing cells to 50°C, 77°C, or H2O2 (Fig. S3), suggesting that caspase-like activities might not be involved in the cell death processes induced by these triggers, in accordance with previous reports in animal cells (Dixon et al., 2012).

Molecular mechanisms governing ferroptosis in *Synechocystis*

To gain insight into the regulation of several candidate genes that might be involved in cyanobacteria ferroptosis, we examined their expression after exposing *Synechocystis* cells to a 50°C-HS, preincubated or not with Fer-1 or CPX (Table 1). The genes analyzed include the following. Genes were analyzed that were involved in cyanobacterial GSH synthesis (*gshA* and *gshB*) and GSH catabolism (*ggt*; Narainsamy et al., 2016); and *gpx1* and *gpx2*, orthologues to human GSH peroxidase (GPX4), which is essential for ferroptosis in animals (Friedmann Angeli et al., 2014; Imai et al., 2017). Three genes, *gshA*, *ggt*, and *gpx2*, were upregulated after 50°C exposure, and only *gpx2* was down-regulated.
On the other hand, gpx1 and gshB were found to be poorly expressed in the analyzed conditions. Only gpx2 showed a clear response to HS, an induction of ∼10-fold that was prevented by CPX (Table 1). Genes were analyzed that were involved in Fe metabolism such as ferric and ferrous iron transporters (futA, futB, futC, and feoB) and the isiA gene, which encodes a chlorophyll-binding protein inducible by high light (Havaux et al., 2005). FeoB was highly up-regulated after the 50°C-HS, which was enhanced by the pretreatment with Fer-1 (Table 1). Genes were analyzed that were related to the heat shock regulon involved in heat stress (groES, groEL; Rajaram et al., 2014). GroES and GroEL were up-regulated after HS, and that was not prevented by pretreatment with ferroptosis inhibitors (Table 1).

**Discussion**

Altogether, our results demonstrate that an iron-dependent, oxidative type of cell death takes place in a photosynthetic prokaryote (cyanobacteria) in response to heat stress. Notably, several hallmarks that characterize ferroptosis in animals and ferroptosis-like in plants (Distéfano et al., 2017; Dixon and Stockwell, 2019) are conserved in this cyanobacterial pathway.

Synechocystis cell death triggered by 50°C-HS is prevented by the canonical ferroptosis inhibitors CPX, Fer-1, Lipro-1, and vitamin E (Dixon and Stockwell, 2019; Kagan et al., 2017; Stockwell and Jiang, 2020). While CPX is a membrane-permeable iron chelator, the other ferroptosis inhibitors act by preventing the production of lipid hydroperoxides, which are the ultimate pro-ferroptotic signals (Friedmann Angeli et al., 2014; Kagan et al., 2017; Stockwell and Jiang, 2020; Anthonymuthu et al., 2021).

Lipid ROS accumulation can also be prevented by supplementing cells with bis-allylic deuterated PUFAs, which are less susceptible to peroxidation (Bogacz and Krauth-Siegel, 2018; Distéfano et al., 2017). Cell death in Synechocystis was significantly reduced when cells were preincubated with deuterated PUFAs, as seen in eukaryotes (Figs. 3 and 4). PUFAs are present in cyanobacteria as part of phospho- and sulfo-lipids in the membranes of thylakoids. Several stress conditions such as high temperature, high light, or algaecide exposure have been shown
Figure 5. Oxidized species of PE, PG, and SQDG are accumulated in response to HS. Quantitative assessments of oxidized (ox) lipid species generated in *Synechocystis* sp. PCC 6803 cells exposed to HS and the ferroptosis inhibitor Fer-1. (a) Typical mass spectrum of PE found in cyanobacteria acquired in negative ion mode.
to trigger the generation of lipid peroxides (Allakhverdiev et al., 1999; Latifi et al., 2009; Lee et al., 2018; Maeda et al., 2005; Singh et al., 2002). Oxidation of PUFA-containing phospholipids by lipoxygenases (LOXs; encoded by the ALOX genes) is a likely mechanism required contributing to the execution of ferroptotic cell death in mammalian cells (Yang et al., 2016). Several LOX sequences have been detected in cyanobacteria from the genera Anabaena, Nostoc, Microcystis, and Synechococcus, and a mini-LOX has been biochemically characterized in Cyanothecae (Andreou et al., 2010; Hansen et al., 2013; Table 2). However, the biological role of bacterial LOXs remains to be elucidated (Hansen et al., 2013), with the notable exception of 15-LOX from a Gram-negative bacterial pathogen, Pseudomonas aeruginosa, that oxidizes host arachidonoyl-PE and triggers “theft-ferroptosis” in a variety of mammalian cells (Dar et al., 2018).

Long-chain acyl-CoA synthetase-4 (ACSL4) modulates the metabolic fates of PUFAs in human and mouse cells. Remarkably, the deletion of the ACSL4 gene causes resistance to ferroptosis in mammals (Doll et al., 2017). The Synechocystis genome includes a sequence that encodes a putative acyl-acyl carrier protein synthetase (SynAas, Slr1609) that is a homologue of the Arabidopsis thaliana long-chain acyl-CoA synthetase 9 (LACS9; Table 2). SynAas specifically uses acyl-acyl carrier protein as a cosubstrate to recycle free fatty acids and it is also involved in the transfer of free fatty acids across membranes by vectorial acylation (Kaczmarzyk and Fulda, 2010; von Berlepsch et al., 2012). slr1609-knockout mutants are not able to import exogenous fatty acids as well as fatty acids secreted from membrane lipids into the culture medium (Kaczmarzyk and Fulda, 2010), highlighting the role of SynAas in recycling fatty acids. Further studies are required to evaluate whether SynAas deletion prevents ferroptosis in Synechocystis as well.

Lipidomic studies have identified oxidized arachidonic/adrenic PEs as pro-ferrototic signals in human cells (Kagan et al., 2017). While in general prokaryotes membranes contain saturated or monounsaturated lipids, the inner membranes of thylakoids contain MGDGs, digalactosyldiacylglycerols, SQDGs, PGs, and PEs with fatty acyl substituents with a variable number of carbon atoms and a high degree of unsaturation (Hewelt-Belka et al., 2020; Wada and Murata, 1998).

Our results demonstrating the Fer-1–inhibitable accumulation of oxidized species of PE, PG, and SQDG in response to HS strongly suggest that these oxidized species might act as pro-ferroptosis signals in cyanobacteria. Remarkably, we found that when cyanobacteria were exposed to 50°C, the cells clearly showed a loss of the thylakoids membrane integrity, with evident nonelectron dense zones, and vesiculation. Notably, these features are already known to occur after heat treatment in plants (Ristic et al., 2007) and in the unicellular eukaryotic algae.

**Figure 6.** Morphological studies of Synechocystis sp. PCC 6803 under heat and oxidative stress. TEM micrographs of Synechocystis sp. PCC 6803 cells. Cultures were treated with DMSO (control), 50°C, or 77°C for 10 min or with H$_2$O$_2$ 10 mM for 1 h.
Table 2. Components of the eukaryotic ferroptosis pathway found in cyanobacteria

| Gene/metabolite/process | Cyanobacteria |
|------------------------|---------------|
| Selenium-containing GSH peroxidase 4 (GPX4) | The genome of Synechocystis sp. PCC 6803 encodes two gpx-like proteins annotated as GPX1 (slr1171) and GPX2 (slr1992) with high similarity to higher plants and mammals. In vitro experiments show that they use unsaturated fatty acid hydroperoxides or alkyl hydroperoxides as electron acceptors (Gaber et al., 2001). |
| FSP1-ubiquinol | Synechocystis sp. PCC 6803 encodes one dihydroorotate dehydrogenase (slr1418) that catalyzes the conversion of dihydroorotate to orotate with quinone or plastoquinone as electron acceptor (Nara et al., 2000; Baers et al., 2019). |
| Dihydroorotate dehydrogenase–ubiquinol | Unknown. BlastP searches using cystine/glutamate transporter [Q9UPY5 (XCT_HUMAN)] as query against cyanobacterial genomes retrieved amino acid permissives (this work). |
| SLC7A11 transmembrane protein, a key component of the cystine/glutamate transporter system xc– | Unknown. BlastP searches using cystine/glutamate transporter [Q9UPY5 (XCT_HUMAN)] as query against cyanobacterial genomes retrieved amino acid permissives (this work). |
| GSH | Key antioxidant involved in the protection against ROS (Latifi et al., 2009). Cell death induced by heat (50°C) is correlated with GSH content in Synechocystis sp. PCC 6803 (Suginaka et al., 1999; this work). External addition of GSH prevents cell death in cultures exposed to heat (50°C; this work). |
| ACYL4 | Unknown. Acyl-acyl carrier protein synthetase of Synechocystis sp. PCC 6803 (SynAas, Slr1609, homologue of Arabidopsis LACS9) recycles free fatty acids, and it is also involved in the transfer of free fatty acids across membranes by vectorial acylation (Kaczmarzyk and Fulda, 2010; von Berlepsch et al., 2012). |
| PUFAs | Generally present in cyanobacterial membranes. The unsaturation of fatty acids in membrane lipids enhances the tolerance to salt stress and is essential for low temperature tolerance in Synechocystis sp. PCC 6803 (Allakhverdiev et al., 1999; Singh et al., 2002). Cell death induced by heat (50°C) is prevented by supplementation with deuterated PUFAs (this work). |
| Lipid peroxidation | Measured in several cyanobacteria under stress conditions (Maeda et al., 2005; Latifi et al., 2009; Lee et al., 2018). |
| LOXs | Unknown. Genes with homology to LOXs (ALOX genes) have been found in some cyanobacteria (Hansen et al., 2013). |
| Vitamin E (α-tocopherol) | Tocopherols have a role in protecting Synechocystis sp. PCC 6803 from lipid peroxidation and high light stress (Maeda et al., 2005). |
| Autophagy | Cyanobacteria have approximately 10 homologues of autophagy genes, which suggests the prokaryotic origin of some autophagy-related proteins (Yang et al., 2016). |

Several key genes, metabolites, and processes relevant to ferroptosis described in humans (Dixon et al., 2012; Conrad et al., 2018) are shown as well as a summary of their potential role in cyanobacteria.

*Chlorella saccharophila* during cell death induced by heat (Zuppini et al., 2007).

The high levels of vacuolization observed in *Synechocystis* cells after exposure to 50°C for 10 min (Fig. 6) could be due to an increase in the autophagic flux. Ferroptosis has been described as an autophagy-dependent process in animal systems (Zhou et al., 2020). While autophagy is present in almost all eukaryotes, little is known about whether this process occurs in cyanobacteria. Most prokaryotes have three or four homologues of autophagy genes, but cyanobacteria carry approximately 10 homologues (Yang et al., 2017; Table 2). Notably, it was reported that endosymbiotic cyanobacteria present in the fern *A. microphylla* exhibit autophagy-like cell death that is characterized by a gradual condensation and degradation of the cytoplasm and high levels of vacuolization (Zheng et al., 2013). These observations suggest that autophagy might also be a part of the ferroptotic mechanism in cyanobacteria, although more studies are needed to establish if that is the case.

Eukaryotic ferroptosis is highly linked to GSH metabolism and content (Dixon and Stockwell, 2019). In cyanobacteria, GSH is a key antioxidant involved in the protection against several ROSs (Latifi et al., 2009). In this work, we showed that cell death induced by 50°C was preceded by the depletion of the antioxidants GSH and AsA (Fig. 3, a–d), as reported previously for *Synechocystis* (Suginaka et al., 1999), mammalian cells, and root hairs of *A. thaliana* (Distefano et al., 2017; Dixon and Stockwell, 2019; Yang and Stockwell, 2016). Moreover, cell death was prevented by preincubation with GSH, AsA, and DTT, suggesting that GSH and AsA depletion is required for HS-induced cell death in *Synechocystis* (Fig. 3, e and f). In mammalian cells, GSH depletion results in the inactivation of the selenoenzyme GPX4, which in turn causes an overwhelming accumulation of lipid peroxides that triggers ferroptosis (Dixon et al., 2012; Seiler et al., 2008; Skouta et al., 2014). Similarly, ferroptosis-like death in plants is characterized by the early depletion of GSH and AsA (Distefano et al., 2017) as also observed in cyanobacteria (Fig. 3). However, the mechanisms through which GSH is depleted in plants and cyanobacteria are not yet well understood. GSH depletion in eukaryotes can be explained by its extensive consumption in the ER lumen to repair disulfides formed as a consequence of high temperatures (Distefano et al., 2017; Ozgur et al., 2014). Although cyanobacteria lack the ER, it was proposed...
that the process could be very similar in these organisms (Chatterjee et al., 2020; Narainsamy et al., 2016). Additionally, when GSH pool is depleted, AsA cannot be recycled via the thiol-dependent mechanisms. This can explain the low levels of reduced AsA detected in *Synechocystis* treated with 50°C and in *Arabidopsis* roots after heat stress (Distéfano et al., 2017; Foyer and Noctor, 2011; Noctor et al., 2012; this work).

GPX4 is a key regulator of eukaryotic ferroptosis (Seibt et al., 2019; Yang et al., 2014). The genome of *Synechocystis* encodes two GPX-like proteins annotated as GPX1 (sll1771) and GPX2 (sll1992), which display high similarity to GPXs of angiosperms and mammals that do not contain selenium (Gaber et al., 2001; Table 2). In vitro experiments have shown that recombinant GPX-like proteins of *Synechocystis* can reduce unsaturated fatty acids using NADPH, but they are unable to use GSH as an electron donor (Gaber et al., 2001). However, cyanobacteria have multiple detoxifying enzymes to manage oxidative stress, such as GSH peroxidases and peroxiredoxins (Johnson and Hug, 2019), that might also be involved in this response. The redox network in plants and cyanobacteria is composed of several specific reductases that transfer electrons to either GSH or thioredoxins (Buchanan and Luan, 2005; Müller-Schussele et al., 2021; Pérez-Pérez et al., 2009). Thus, GPXs in cyanobacteria could use thioredoxins as electron donors, as seen in plants (Gaber et al., 2012).

An alternative system that suppresses phospholipid peroxidation and confers protection against ferroptosis independently from GPX4 and GSH has been recently described in mammalians cells (Doll et al., 2019; Mao et al., 2021). The flavoprotein apoptosis-inducing factor mitochondria-associated 2 (renamed ferroptosis suppressor protein 1 [FSP1]) is a NAD(P)H-dependent oxidoreductase involved in cellular oxidative stress response that counteracts ferroptosis by generating ubiquinol from ubiquinone (also known as coenzyme Q10; Doll et al., 2019). In addition, the enzyme dihydroorotate dehydrogenase was shown to convert ubiquinone to ubiquinol exclusively in mitochondria, helping to combat the effects of lipid peroxidation in a mechanism that resembles the FSP1 system in the plasma membrane (Mao et al., 2021). In plants, plastoquinone and ubiquinone are electron transporters in the electron transport chain of photosynthesis and the aerobic respiratory chain, respectively. Importantly, their reduced forms (plastoquinol and ubiquinol) act as radical scavenging antioxidants to prevent lipid peroxidation, protein oxidation, and DNA damage in the plant response to biotic and abiotic stresses (Havaux, 2020; Liu and Lu, 2016). Although less studied, some components of the photosynthetic electron transport chain have been shown to be important for tolerating oxidative stress in cyanobacteria (Latifi et al., 2009). Plastoquinone is present in cyanobacterial thylakoids and is an essential electron carrier required for photosynthesis and respiration (Mullineaux, 2014). Recent studies suggest that ubiquinol biosynthesis originated in this prokaryotic group (Deggli Esposti, 2017). However, it is not known whether plastoquinone or ubiquinone prevents lipid peroxidation in cyanobacteria. On the other hand, the genome of *Synechocystis* encodes one membrane-bound dihydroorotate dehydrogenase (pyrD, sll1418) that catalyzes the conversion of dihydroorotate to orotate with quinone or plastoquinone as the electron acceptor (Baers et al., 2019; Nara et al., 2000; Table 2). Further studies are required to elucidate if these enzymes and antioxidants also confer protection against cyanobacterial ferroptosis.

The role of calcium in ferroptosis is still a matter of debate. While the extracellular calcium chelator EGTA was found to block ferroptosis in plants (Distéfano et al., 2017), chelation of extracellular calcium does not prevent cell death in response to GSH depletion in human cancer cells (Dixon et al., 2012). Another report, however, indicates that extracellular calcium influx is required for cell death downstream of GSH depletion in mammalian neuronal-like HT22 cells (Henke et al., 2013). These different behaviors suggest that calcium requirements could be cell type-specific. Calcium is an important second messenger in cyanobacteria and is involved in responses to several stresses such as temperature shock and osmotic stress (Agostoni and Montgomery, 2014). Our studies using calcium chelators and exogenous calcium addition (Fig. 2) suggest that extracellular calcium influx can prevent cell death, in agreement with previous results showing that exogenous Ca²⁺ supplementation improves the tolerance of *Anabaena* sp. PCC 7120 to heat stress (Tiwari et al., 2016). In *Anabaena* sp. PCC7120, a heat shock of 45°C results in a significant increase of intracellular Ca²⁺ levels. Interestingly, the use of Ca²⁺ chelators like EGTA or verapamil, a Ca²⁺ channel blocker, suggests that heat shock mobilizes cytosolic Ca²⁺ from both intracellular and extracellular sources (Torrecilla et al., 2000). However, Ca²⁺ from intracellular reservoirs seems to trigger cell death, as incubation with BAPTA-AM prevents ferroptosis triggered by HS. This role for calcium is in agreement with previous reports in eukaryotic cells, particularly in dopaminergic cells (Do Van et al., 2016), where BAPTA-AM prevents ferroptosis induced by erastin. The accumulation of ROS in the cytosol as a result of HS can not only activate redox-sensitive calcium channels but also trigger Ca²⁺ release from the ER, probably through PLC-induced generation of the second messenger inositol 1,4,5-triphosphate. The resultant increase in intracellular Ca²⁺ levels affect several aspects of cellular homeostasis, including mitochondrial membrane potential. In conclusion, although our results support a role for Ca²⁺ as a second messenger in c-ferroptosis as occurs in some eukaryotic death pathways (Clapham, 2007; Decrock et al., 2011; Do Van et al., 2016; Ren et al., 2021), the specific sources of intracellular Ca²⁺ and their function in the execution of c-ferroptosis are still unknown.

**Concluding remarks**

Based on the results obtained in this study, we summarized our current understanding of the pathways leading to ferroptosis in the prokaryote *Synechocystis* (Fig. 7). Concisely, the pathway involves early GSH depletion following an HS, which leads to lipid peroxidation, mostly of PUFA lipids of thylakoid membranes. Canonical ferroptosis inhibitors like the iron chelator ciclopirox CPX, the lipophilic antioxidant Fer-1, Lipro-1, and vitamin E prevent the accumulation of toxic oxidized lipids and cytosolic ROS, inhibiting ferroptotic cell death. Mechanistically, this pathway shows remarkable similarities to eukaryotic...
ferroptosis. Interestingly, thylakoid membranes seem to be involved in this process, possibly providing PUFA lipids that undergo peroxidation. Thylakoid membranes are enriched in PUFAs, which differentiates cyanobacteria from other prokar-yotes, which in general carry membranes containing saturated or monounsaturated lipids (Sharathchandra and Rajashekhar, 2011). This fact can also be related to previous results obtained in the model plant A. thaliana, where active chloroplasts were shown to contribute to ferroptotic cell death in leaves (Distéfano et al., 2017).

Altogether, these findings open up a broad field of future research investigating cell death in cyanobacteria, which could be of great relevance in the management of toxic blooms and their ecological consequences.

**Materials and methods**

**Cyanobacterial cells and culture conditions**

Axenic cultures of *Synechocystis* sp. PCC 6803 were grown in an orbital shaker (120 rpm) at 28 ± 2°C, under constant light (30 µE m⁻² s⁻¹) in BG11 medium buffered with 20 mM HEPES-KOH to pH 7.5 (Rippka et al., 1979).

**Pre-incubations and heat treatments**

Cultures growing in control condition were submitted to heat treatments in a water bath when they reached logarithmic phase (approximately OD₇₅₀ 0.8–0.9). Cells were exposed to 50°C or 77°C for different times (10 min, 30 min, 60 min, 2 h, 4 h, and 6 h). GSH (L-GSH reduced form; Sigma-Aldrich; final concentration 100 µM), AsA (L-AsA; Merck; final concentration 1 µM), D-PUFAs (Retrotope; final concentration 50 µM), CPX (final concentration 1 µM), Fer-1 (final concentration 1 µM), Lipro-1 (final concentration 5 µM) and vitamin E (final concentration 500 µM) were applied to *Synechocystis* sp. PCC 6806 cultures 24 h before the HS. A second pulse of Fer-1 (1 µM) was added 2 h before the HS. For Cl₂Ca, EGTA (extracellular calcium chelator) and BAPTA-AM (intracellular calcium chelator; Torrecka et al., 2004) experiments, compounds were applied as follows: (1) Cl₂Ca (3 mM final concentration) 24 h before HS; (2) EGTA (1 mM final concentration) 24 h before HS; (3) Cl₂Ca (3 mM) for 2 h followed by addition of EGTA (1 mM) 22 h before HS (Distéfano et al., 2017); and (4) EGTA for 2 h followed by incubation with Fer-1 or CPX for 22 h before inducing cell death by treating cells at 50°C for 4 h. Cultures with no addition were used as controls.

**Drop tests for viability analysis**

The effect of H₂O₂ (2, 5, and 10 mM for 1 h) and HS (50°C and 77°C for 10 min) in the absence or presence of Fer-1, CPX, GSH, AsA, EGTA, and CaCl₂ was tested on solid medium. Serial dilutions of treated cultures were prepared (10⁶–10⁻³), spotted onto solid BG11 agar plates, and cultivated under constant light (30 µE m⁻² s⁻¹) at 26 ± 2°C for 20 d.

**Cell counting and viability determination**

*Synechocystis* cell suspensions were stained with SYTOX Green at a final concentration of 1 µM for 10 min protected from light. Cells were examined and counted by light and fluorescence microscopy using a Nikon E600 microscope equipped with a B-2A cube with 450–490-nm excitation and 500–515-nm emission filters, and a Neubauer chamber. Images were captured by Olympus DP72 digital camera, using Cellsens Entry imaging software. At least 10 random fields were taken for viability calculations in each experiment and quantified in Image J (https://imagej.nih.gov/ij/; Schulze et al., 2011; Fig. S3, a and b).

**TEM analysis**

*Synechocystis* cells subjected to H₂O₂ (10 mM for 1 h) and HS (50°C and 77°C for 10 min) were fixed with 2.5% (vol/vol) glutaraldehyde in PBS buffer overnight at 4°C. After washing three times in PBS buffer, samples were post-fixed with 1% (vol/vol) osmium tetroxide in medium buffer for 1 h and washed twice in distilled water. Samples were dehydrated in increasing concentrations of alcohol and embedded in Spurr epoxy. Ultrathin sections (90 nm) were stained in uranyl acetate and lead citrate and examined with a JEM 1200 EX II transmission electron microscope (JEOL Ltd.). Images were captured using a digital camera (Erlangshen ES 1000 W, model 785) from the Central Service of Electron Microscopy of the Faculty of Veterinary Sciences, Universidad Nacional de La Plata (Argentina).

**Measurement of GSH and AsA content**

50 ml of exponentially growing culture (~2.5 × 10⁷ cells ml⁻¹) were collected by centrifugation (9,300 × g for 10 min, 4°C), resuspended in 1 ml 3% trifluoracetic acid, mixed thoroughly
(vortexed for 1 min), frozen with liquid nitrogen, thawed in ice, and mixed in vortex for 1 min prior to centrifugation (16,000 × g for 15 min, 4°C). Supernatants were passed through a C-18 column (Bond Elute; Varian) for a partial sample purification and eluted in 1 ml 100 mM phosphate buffer, pH 7. Then supernatant was used for GSH and AsA determinations.

The determination of reduced AsA and dehydroascorbate (the oxidized form of AsA) was performed following Aguilera et al. (2020). The content of AsA was determined with a HPLC system using an UV-VIS detector (Model SPD-10AV; Shimadzu) at λ = 265 nm coupled with a LC-10 AT pump (Shimadzu) and compounds separated in a C-18 column (Microsphere C-18 SS 100 × 4.6 mm; Varian Inc.). Results were expressed in picomoles of AsA per cell.

Determination of GSH and GSSG was done following the 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) method using GSH reductase and 2-vinylpyridine following Aguilera et al. (2020). Results were expressed in micromoles of GSH or GSSG per cell per mg of fresh weight basis, and in nanomoles per cell.

**Flow cytometry experiments**

Cell viability, ROS production, lipid peroxidation, and caspase-like activity were assessed by flow cytometry in a Partec Cyflow Space cytometer equipped with a 488-nm laser (blue) and three detectors: 525 nm (green), 590 nm (orange), and 675 nm (red). *Synechocystis* cells in logarithmic phase (OD = 0.8) were used in all the assays.

To test viability, FDA was used as it enters and emits fluorescence only in living cells (Gumbo et al., 2014). An FDA stock solution (1000X) was prepared by solubilizing 50 mg of FDA in 5 ml of DMSO and stored in the dark at −20°C until further use. Cultures pretreated or not with Fer-1 and CPX for 24 h were subjected to 50°C and 77°C for 10 min. After 16 h, cultures were incubated with FDA for 10 min. General cytosolic ROS was measured using H2DCFDA. A 10 mM H2DCFDA stock solution was prepared in DMSO and stored in the dark at −20°C. BODIPY C-11 581/591 (undecanoic acid) was used for the analysis of lipid peroxidation. This probe detects the oxidation of the polyunsaturated butadienyl portion, changing its emission peak from ∼590 nm to ∼510 nm (Cheloni and Slaveykova, 2013). A 2 mM (1000X) BODIPY stock was prepared and frozen at −20°C. Cultures pretreated or not with Fer-1 or CPX for 24 h were subjected to 50°C and 77°C for 10 min. For ROS and lipid peroxidation analysis, aliquots were taken at different times (30 min, 1 h, 2 h, and 3 h) after HS, incubated with H2DCFDA or BODIPY for 30 min, and analyzed in the cytometer. Aliquots treated with 500 mM H2O2 were used as a positive control for the formation of ROS and oxidized lipids. Caspase-like activity was measured using CellEvent Caspase-3/7 Green Flow Cytometry Assay Kit (Invitrogen), a nucleic acid-binding dye thatharbors the caspase-3/7 cleavage sequence, DEVD, and is fluorescent after being cleaved and bound to DNA. Analysis of raw flow cytometry data was done with FlowJo (https://www.flowjo.com/).

**Oxidative lipodomics**

Lipids were extracted by Folch procedure (Folch et al., 1957) with slight modifications, under nitrogen atmosphere at all steps. LC-electrospray ionization–mass spectrometry (LC/ESI-MS) analysis of lipids was performed on a Dionex HPLC system coupled to an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific). Major lipid classes were separated on a normal phase column (Luna 3 μm Silica [2] 100 A; 150 × 1.0 mm; Phenomenex) at a flow rate of 0.050 ml/min. The column was maintained at 35°C. The analysis was performed using gradient solvents as previously described (Sun et al., 2021). To quantitatively assess oxidized molecular species, lipids were separated on a C30 reverse phase column (Accucore; 2.1 mm × 25 cm; 2.6 μm particle size; Thermo Fisher Scientific). Solvent A was acetonitrile/water (50/50); solvent B was 2-propanol/acetonitrile/water (85/10/5). Both A and B solvents contained 5 mM ammonium formate and 0.1% formic acid as modifiers. The gradient method was as follows: 0–40 min, 15–50% B (linear, 5); 40–130 min, 50–100% B (linear, 5); 130–150 min, hold at 100% B; 150–140 min, 15% B (linear, 5); 140–150 min, 15% B for equilibration. The flow was maintained at 100 μl/min. The LC system was a Thermo Ultimate 3000 complete with a WPS-3000 autosampler. Column temperature was set at 35°C. Analysis of LC with mass spectrometry data was performed using software package Compound Discoverer (Thermo Fisher Scientific) with an in-house-generated analysis workflow and lipid database. Lipids were further filtered by retention time and confirmed by fragmentation mass spectrum. Deuterated lipids (Avanti Polar Lipids) were used as internal standards.

**RNA isolation and quantitative real-time RT-PCR**

Before carrying out our experiments, we examined the expression profiles of our genes of interest (e.g., genes potentially involved in ferroptosis such as cyanobacterial GSH synthesis, Fe metabolism, heat shock response) on expression data (microarray measurements and RNA sequencing) available at CynoEXpress (http://193.136.227.175/cyanox/cyanoexpress.html; files/cyanoexpress.intro.html). CynoEXpress is a web server that enables interactive exploration of curated genome-wide expression data for *Synechocystis* sp. PCC 6803 (Hernandez-Prieto and Futschik, 2012). In particular, we used the RNA sequencing data containing the gene expression of *Synechocystis* sp. PCC 6803 exposed to heat stress of 42°C for 30 min (Kopf et al., 2014). This exploratory analysis showed that several of our genes of interest were up-regulated right after the heat stress. Based on that, we selected the time points to assess gene expression (60 min after the heat shock) in our experiments. Target genes were identified in the genome of *Synechocystis* (http://genome.annotation.jp/cyanobase/Synechocystis), and PCR primers were designed with Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast; Table S1). The analyses of gene expression were performed by real-time quantitative PCR (qPCR). Total RNA was extracted from cell pellets using TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions. After digestion with RQ1 RNase-free DNase (Promega), RNA (1 μg) was retro-transcribed using random hexamers (Promega). The qPCR reactions were performed in a Step One real-time PCR system (Applied Biosystems) using a Micro Amp Fast Optical 48-well reaction plate with 15 μl reaction volume containing 1× Power Sybr Green PCR Master Mix (Thermo Fisher Scientific).
Fisher Scientific), 0.2 µM of each primer, and 1.5 µg of cDNA. The cycling program was 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 15 s, and 40 cycles of 54°C for 1 min. A melting curve analysis was conducted to verify the formation of a single unique product and the absence of potential primer dimerization. The different biological samples were subsequently normalized against expression of mpB gene coding for the RNA subunit of RNaseP.

Data analysis
All experiments were performed at least in triplicate. The results are expressed as the mean ± SD. The main effects of treatments were examined by running two-way ANOVA for AsA and GSH data, and two-way ANOVA in generalized linear models (GLMs) module for the rest of the experiments. Binomial distribution was fitted using the glm function in R. The Shapiro–Wilk test was used to test whether AsA and GSH data (two-way ANOVA) and residuals for the rest of experiments (two-way ANOVA in GLM) were normally distributed. When significant differences (P < 0.05) were found, the Tukey post hoc test was used for multiple comparisons within groups. Statistical analyses were performed using the open access software R (https://www.R-project.org). The ANOVAs of the GLM models and post hoc comparisons were performed with car and lsmeans packages (Fox and Weisberg, 2010; Lenth, 2016).

Online supplemental material
Fig. S1 presents representative fluorescence images showing cell death in Synechocystis sp. PCC 6803. Fig. S2 shows caspase-like activity measured by flow cytometry using CellEvent after 50°C and 77°C for 10 min, or with H2O2 10 mM for 1 h. Fig. S3 shows the content of MGDG species in cyanobacteria. Table S1 lists primers used in this study in qPCR reactions.

Acknowledgments
We thank Mikhail S. Shchepinov of Retrotepe, Inc., Los Altos, CA, for providing D-PUFAs and Natalia Correa-Aragunde of Instituto de Investigaciones Biológicas-Consejo Nacional de Investigaciones Científicas y Técnicas, Universidad Nacional de Mar del Plata, Mar del Plata, Argentina for providing BAPTAM. We would like to thank Silvana Colman, Macarena Perez-Cenci, Gonzalo Caló, Natalia Almada, and Viviana Daniel for technical assistance and Daniela Suelo and Juan José Gueime for insightful comments.

This research was funded by grants from Agencia Nacional de Promoción Científica y Técnica Argentina (PICT 1956 and PICT 0173 to M.V. Martin and PICT-2017-0201 to G.C. Pagnussat) and International Centre for Genetic Engineering and Biotechnology (ARG 19-06). A. Aguilera is a postdoctoral fellow of Consejo Nacional de Investigaciones Científicas y Técnicas; F. Berdun is a fellow of Consejo Interuniversitario Nacional. M.V. Martin, G.C. Pagnussat, G. Salerno, and C.G. Bartoli are Consejo Nacional de Investigaciones Científicas y Técnicas researchers.

The authors declare no competing financial interests.

Author contributions: Conceptualization: M.V. Martin, G. Pagnussat, A. Aguilera, F. Berdun, and G. Salerno; methodology: M.V. Martin, G. Pagnussat, A. Aguilera, F. Berdun, C. Bartoli, C. Steelheart, M. Alegre, H. Bayir, Y.Y. Tyurina, and V.E. Kagan; investigation: M.V. Martin, A. Aguilera, F. Berdun, G. Pagnussat, G. Salerno, H. Bayir, Y.Y. Tyurina, and V.E. Kagan; formal analysis: M.V. Martin, A. Aguilera, F. Berdun, G. Pagnussat, H. Bayir, Y.Y. Tyurina, and V.E. Kagan; funding acquisition: M.V. Martin and G. Pagnussat; validation and statistical analysis: M.V. Martin, G. Pagnussat, A. Aguilera, F. Berdun, H. Bayir, Y.Y. Tyurina, and V.E. Kagan; writing of the original draft: M.V. Martin, G. Pagnussat, A. Aguilera, F. Berdun, G. Salerno, V.E. Kagan, and C. Bartoli.

Submitted: 1 November 2019
Revised: 29 September 2021
Accepted: 5 November 2021

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Viability assays. (a) Representative fluorescence images showing cell death in Synechocystis sp. PCC 6803 detected by SYTOX Green nucleic acid stain. (b) Neither Fer-1 nor CPX prevented cell death triggered by H$_2$O$_2$ treatments. (c) Kinetics of cell death induced by 50°C. (d) GSH (100 µM) and AsA (1 µM) addition prevents cell death induced by 50°C HS (10 min). (a–d) Cultures were preincubated with DMSO, Fer-1 (1 µM), or CPX (1 µM) for 24 h before HS. (b and d) Viability was tested via drop test. Scale bars, 5 µm.
One table is provided online as a separate file. Table S1 shows primers used in this study in qPCR reactions.

Figure S2. Caspase-like activity measured by flow cytometry using CellEvent after 50°C, 77°C for 10 min, or with H₂O₂ 10 mM for 1 h.

Figure S3. Content of MGDG species in cyanobacteria. Data are presented as pmol/mg protein. Statistic: one-way ANOVA. Separation and quantification of lipid was performed by using reverse phase chromatography. MGDG species were detected as acetate adducts. *, P < 0.05 versus control; **, P < 0.05 versus ΔwspF (one-way ANOVA); n = 5.