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## The bZIP1 Transcription Factor Regulates Lipid Remodeling and Contributes to ER Stress Management in *Chlamydomonas reinhardtii*

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### ► To cite this version:

Yasuyo Yamaoka, Seungjun Shin, Bae Young Choi, Hanul Kim, Sunghoon Jang, et al.. The bZIP1 Transcription Factor Regulates Lipid Remodeling and Contributes to ER Stress Management in *Chlamydomonas reinhardtii*. The Plant cell, 2019, 31 (5), pp.1127-1140. 10.1105/tpc.18.00723 . cea-02134206

**HAL Id: cea-02134206**

**<https://cea.hal.science/cea-02134206v1>**

Submitted on 17 Feb 2020

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1 **The bZIP1 Transcription Factor Regulates Lipid Remodeling and**  
2 **Contributes to ER Stress Management in *Chlamydomonas reinhardtii***

3  
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25  
26 *The authors declare there is no conflict of interests.*

27  
28 **Running title:** CrbZIP1 modulates membrane lipids under ER stress

29  
30 **One-Sentence Summary:** The mRNA of a *Chlamydomonas* bZIP transcription factor is  
31 spliced by CrIRE1 under ER stress, and the resulting protein protects *Chlamydomonas* cells  
32 from ER stress by modulating lipid remodeling.

33  
34  
35 **Keywords:** bZIP; *Chlamydomonas*; DGTS; ER stress; pinolenic acid

36  
37 **Word count:** 7958 words

40 **ABSTRACT**

41 Endoplasmic reticulum (ER) stress is caused by the stress-induced accumulation of unfolded  
42 proteins in the ER. Here, we identified proteins and lipids that function downstream of the  
43 ER stress sensor inositol-requiring enzyme1 (CrIRE1), which contributes to ER stress  
44 tolerance in *Chlamydomonas reinhardtii*. Treatment with the ER stress inducer tunicamycin  
45 resulted in the splicing of a 32-nucleotide fragment of a bZIP transcription factor (*CrbZIP1*)  
46 mRNA by CrIRE1, which resulted in the loss of the transmembrane domain in CrbZIP1, and  
47 the translocation of CrbZIP1 from the ER to the nucleus. Mutants deficient in *CrbZIP1* failed  
48 to induce the expression of the unfolded protein response genes and grew poorly under ER  
49 stress. Levels of diacylglyceryltrimethylhomo-Ser (DGTS) and pinolenic acid (18:3 $\Delta$ 5,9,12)  
50 increased in the parental strains but decreased in the *crbzip1* mutants under ER stress. A yeast  
51 one-hybrid assay revealed that CrbZIP1 activated the expression of enzymes catalyzing the  
52 biosynthesis of DGTS and pinolenic acid. Moreover, two independent alleles of *crdes* mutant,  
53 which failed to synthesize pinolenic acid, were more sensitive to ER stress than were their  
54 parental lines. Together, these results indicate that *CrbZIP1* is a critical component of the ER  
55 stress response mediated by CrIRE1 in *Chlamydomonas* that acts via lipid remodeling.

56

## 57 INTRODUCTION

58 *Chlamydomonas reinhardtii* is a model microalga widely used for studies of photosynthesis,  
59 flagella biogenesis, and lipid metabolism (Harris, 2001). Similar to many other microalgae,  
60 *Chlamydomonas* produces a large amount of triacylglycerol (TAG) under stress conditions  
61 (Merchant et al., 2012), and this prompted intensive studies of the mechanisms underlying  
62 stress responses in this organism. The endoplasmic reticulum (ER) stress response is an  
63 important stress response that is common to many eukaryotes. ER stress can be induced by  
64 biotic and abiotic factors, such as pathogen infection, temperature, high salinity, wounding,  
65 and ER stress inducing agents (Howell, 2013), and results in the accumulation of unfolded  
66 proteins in the ER. In mammals, the unfolded proteins accumulated in the ER are sensed by  
67 proteins in the ER membrane that transmit this information to the nucleus, inducing the  
68 expression of genes and restoring normal protein metabolism. This process is called the  
69 unfolded protein response (UPR). UPR genes encode molecular chaperones, folding enzymes,  
70 lipid biosynthesis enzymes, and components of the ER-associated protein degradation  
71 pathway (Ron and Walter, 2007).

72         Recently, lipid metabolism was also found to be altered in many types of cells  
73 exposed to ER stress. In animal cells, ER stress increases *de novo* lipogenesis and TAG  
74 accumulation (reviewed in Basseri and Austin 2012; Mandl et al 2013; Zhou and Liu 2014;  
75 Han and Kaufman 2016). In animal and yeast cells, exposure to ER stress promotes the  
76 biosynthesis of phosphatidylcholine (PC), a major ER membrane lipid, expanding the ER  
77 membrane area (Sriburi et al., 2007; Schuck et al., 2009) and thereby attenuating the damage  
78 caused by the stress. The biosynthesis and storage of TAG in lipid droplets might also  
79 ameliorate the damage caused by various stress conditions, since stress often leads to the  
80 degradation of membrane lipids and the release of free fatty acids to toxic levels  
81 (Listenberger et al., 2003). However, little is known about the changes in other lipid  
82 molecules that occur during ER stress. Thus, it would be useful to determine the entire lipid  
83 profile of a cell, to monitor how it changes in response to ER stress, and to establish how it  
84 differs in mutants that do not have a normal ER stress response.

85         The *C. reinhardtii* genome encodes only one ER stress sensor, i.e.,  
86 inositol-requiring enzyme 1 (CrIRE1). IRE1 is the best conserved ER stress sensor among  
87 metazoans, yeast, and plants (Yamaoka et al., 2018). IRE1 is a dual-function protein that has  
88 both kinase and ribonuclease (RNase) activity. Under ER stress, IRE1 dimerizes and then  
89 auto-phosphorylates, triggering its RNase activity. Active IRE1 splices mRNA encoding a

90 transcription factor named *Hac1* in budding yeast, *XBP1* (*X-box binding protein-1*) in  
91 metazoans, and *bZIP60* in *Arabidopsis thaliana* (Kimata and Kohno 2011; Ron and Walter,  
92 2007; Howell, 2013).

93 CrIRE1 plays an essential role in ER stress tolerance in *Chlamydomonas*, similar to  
94 IRE1s of other organisms, but differs from other IRE1s in the C-terminal tandem zinc-finger  
95 domain (Yamaoka et al., 2018). The conserved kinase and RNase domains of CrIRE1 suggest  
96 that CrIRE1, like other IRE1 proteins, splices the mRNA of a transcription factor; however,  
97 the identity of this transcription factor was hitherto unknown in *Chlamydomonas*.

98 In this study, we identified a transcription factor, CrbZIP1, that governs the ER  
99 stress response in *Chlamydomonas*, and revealed the underlying molecular mechanism. In  
100 addition to providing insight into the mode of action of CrbZIP1, this study highlights the  
101 importance of membrane lipid remodeling in ER stress management.

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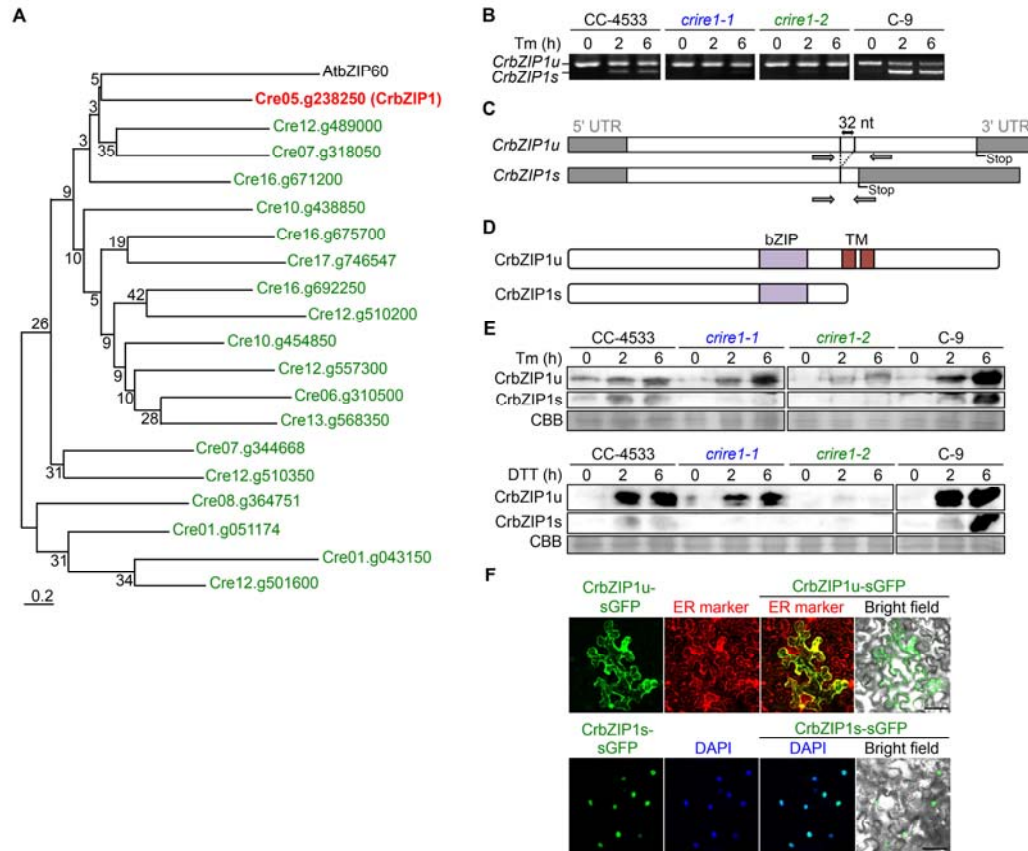
104 **RESULTS**

105 ***Chlamydomonas bZIP1* mRNA is spliced under ER stress**

106 We searched for the mRNA targeted by CrIRE1 from among *Chlamydomonas* basic leucine  
107 zipper (bZIP) proteins, since IRE1 proteins of diverse organisms activate bZIP transcription  
108 factors by splicing their mRNA (Kimata and Kohno 2011; Ron and Walter, 2007; Howell,  
109 2013). Among the 19 genes that contain a bZIP domain in the *Chlamydomonas* genome, we  
110 decided to study Cre05.g238250 (*CrbZIP1*) as the potential target gene of CrIRE1, as it was  
111 the closest relative of *Arabidopsis AtbZIP60* (Figure 1A), the established target of AtIRE1  
112 (Nagashima et al., 2011; Deng et al., 2011).

113 To test whether *CrbZIP1* was indeed the target gene of CrIRE1, we first tested  
114 whether the *CrbZIP1* transcript was spliced under ER stress conditions. Under normal  
115 conditions, there was only one product of *CrbZIP1* transcription, but under the ER stress  
116 induced by tunicamycin, there were two different *CrbZIP1* transcripts in the *Chlamydomonas*  
117 strains CC-4533 (*cw15 mt<sup>-</sup>*, cell wall-deficient) and C-9 (CC-5098, *mt<sup>-</sup>*) (Figure 1B).  
118 However, in the CrIRE1 knock-down mutants *crire1-1* and *crire1-2*, whose parental strain is  
119 CC-4533 (Yamaoka et al., 2018), the smaller transcript was barely detectable (Figure 1B).  
120 Sequencing of the transcripts revealed that 32 nucleotides were spliced out of the *CrbZIP1*  
121 transcript under ER stress, resulting in a frameshift and generating a new stop codon (Figure  
122 1C). We designated the un-spliced form of *CrbZIP1* as *CrbZIP1u* and the spliced form of  
123 *CrbZIP1* as *CrbZIP1s*. The conserved nucleotide sequence commonly found at the splicing  
124 site in the stem-loop structure of IRE1-target mRNA (*AtbZIP60*, *HAC1*, *XBPI* shown in  
125 Supplemental Figure 1) did not exist in the double stem-loop of *CrbZIP1* mRNA predicted  
126 using CentroidFold (Supplemental Figure 1 *CrbZIP1*; <http://rtools.cbrc.jp/centroidfold/>),  
127 suggesting that *Chlamydomonas* IRE1 uses a different mechanism to recognize and splice the  
128 target mRNA.

129 We then compared the predicted structures of proteins encoded by *CrbZIP1u* and  
130 *CrbZIP1s*. Both were predicted to contain a bZIP domain, but only *CrbZIP1u* contained a  
131 transmembrane domain (Figure 1D). The new stop codon generated by splicing was predicted  
132 to delete the transmembrane domain (Figures 1C and 1D). In *Arabidopsis*, *AtbZIP60* mRNA  
133 is spliced by IRE1A/IRE1B at the site of the double stem-loop structure (Supplemental  
134 Figure 1), which causes a frameshift and consequent deletion of a transmembrane domain in  
135 *AtbZIP60* (Nagashima et al., 2011; Deng et al., 2011). Then, the transcription factor is no  
136 longer located at the ER membrane, but enters the nucleus and regulates the expression of  
137 ER-stress-related genes. To test whether *CrbZIP1* undergoes similar modification under ER



**Figure 1. Identification of the transcription factor targeted by *Chlamydomonas* IRE1.**

(A) Molecular phylogenetic analysis of the *Chlamydomonas* bZIP proteins and the *Arabidopsis* AtbZIP60 protein reported to be important for ER stress (Nagashima et al., 2011; Deng et al., 2011). The analysis was conducted in MEGA7 (Kumar et al., 2016), using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). The tree is drawn to scale, with branch lengths measured in number of substitutions per site.

(B) Unspliced *CrbZIP1* (*CrbZIP1u*) and spliced *CrbZIP1* (*CrbZIP1s*) detected using primers flanking the spliced region of *CrbZIP1* (arrows in C). Under the ER stress conditions imposed by 1  $\mu$ g/mL tunicamycin (Tm), *CrbZIP1u* is spliced to *CrbZIP1s* in CC-4533 and C-9, but not in the *crir1* mutants, *crir1-1* and *crir1-2*. *Chlamydomonas* cells were incubated in TAP medium containing 1  $\mu$ g/mL Tm.

(C) Schematic representations of *CrbZIP1u* mRNA and *CrbZIP1s* mRNA and primer locations used to detect splicing.

(D) Schematic representations of primary structures of *CrbZIP1u* and *CrbZIP1s* proteins. The bZIP motif and the transmembrane domain are indicated by bZIP and TM, respectively.

(E) Immunoblot analysis of *CrbZIP1u* and *CrbZIP1s* using anti-*CrbZIP1* antibody. Protein preparations from cells treated with 1  $\mu$ g/mL tunicamycin (Tm) or 5 mM dithiothreitol (DTT) for the indicated periods were subjected to immunoblot analysis. Coomassie brilliant blue staining (CBB) was used as a loading control.

(F) Cellular localization of *CrbZIP1u*-sGFP and *CrbZIP1s*-sGFP transiently expressed in *N. benthamiana* epidermal cells. The superfolder GFP (sGFP), ER marker (AtBiP1-tagRFP), DAPI (nuclear marker), and merged images of sGFP and ER marker, DAPI, and the bright field. Note that *CrbZIP1u*-sGFP mainly localizes to the ER membrane and *CrbZIP1s*-sGFP localizes to the nucleus. Typical images of each localization pattern were chosen from each of the 6 images analyzed. Bars = 50  $\mu$ m.

138 stress, we analyzed the size and amount of *CrbZIP1u* and *CrbZIP1s* proteins under normal  
 139 conditions and the ER stress conditions induced by treatment with 1  $\mu$ g/mL tunicamycin by  
 140 immunoblotting with anti-*CrbZIP1* antibody (Figure 1E, Supplemental Figure 2A). Under  
 141 normal conditions, *CrbZIP1* protein bands were barely detectable, but after ER stress  
 142 treatment, both *CrbZIP1u* and *CrbZIP1s* proteins were detectable in the protein preparations  
 143 from both CC-4533 and C-9 cells (Figure 1E, Supplemental Figure 2A). In addition to  
 144 tunicamycin, treatment with another ER stress agent, dithiothreitol (DTT), also induced both  
 145 *CrbZIP1u* and *CrbZIP1s* proteins in the CC-4533 and C-9 cells (Figure 1E, Supplemental

146 Figure 2A). However, in the *crire1* mutants, only a CrbZIP1u protein band, but not a  
147 CrbZIP1s protein band, was detected (Figure 1E, Supplemental Figure 2A). Thus, the  
148 *CrbZIP1* spliced under ER stress might be the downstream target mRNA of CrIRE1.

149 CrbZIP1 protein levels increased under the ER stress conditions imposed by both  
150 tunicamycin and DTT (Figure 1E, Supplemental Figure 2A); the CrbZIP1u protein level,  
151 which was very low or absent under normal conditions (0 h in Figure 1E and Supplemental  
152 Figure 2A), increased after treatment with the ER stress inducers (2 and 6 h in Figure 1E and  
153 Supplemental Figure 2A). This rapid and large induction of CrbZIP1 proteins was in contrast  
154 to the stable level of *CrbZIP1u* transcript (Figure 1B) and suggested that ER stress induces  
155 the translation of CrbZIP1u protein. Similar regulation of translation was reported in animal  
156 cells; the translation of the *XBP1u* mRNA, the target mRNA of human IRE1, is paused at the  
157 N terminus of XBP1 under normal conditions but resumes rapidly under ER stress  
158 (Yanagitani et al., 2009; Yanagitani et al., 2011).

159

#### 160 **Spliced CrbZIP1s is translocated to the nucleus from the ER**

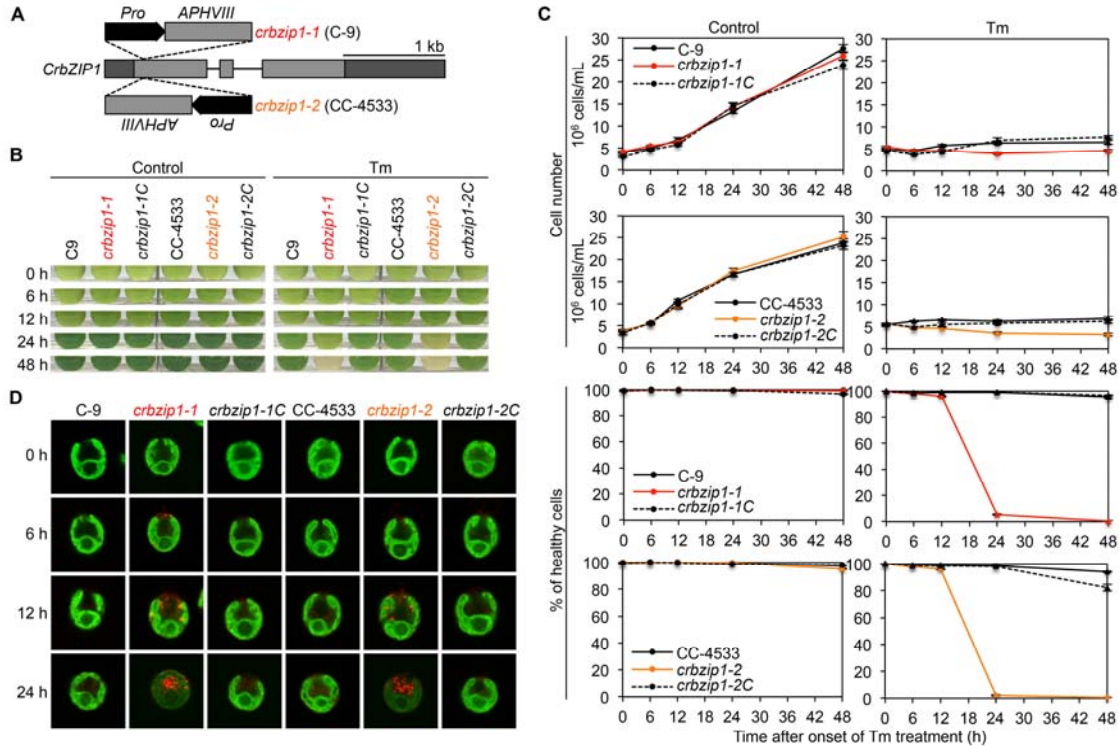
161 Next, to test whether the splicing of *CrbZIP1u* mRNA, which was predicted to remove the  
162 transmembrane domain, altered the subcellular localization of CrbZIP1s protein, we  
163 transiently expressed CrbZIP1 proteins fused with superfolder GFP (sGFP) at their C-termini  
164 in *Nicotiana benthamiana* epidermal cells. CrbZIP1u-sGFP colocalized with the ER marker  
165 AtBiP1-tagRFP, whereas CrbZIP1s-sGFP colocalized with the nucleus marker DAPI (Figure  
166 1F). Together, these results suggest that ER stress activates the RNase activity of CrIRE1,  
167 which splices *CrbZIP1u* transcript to *CrbZIP1s*, and the resulting CrbZIP1s localizes to the  
168 nucleus, consistent with its function as a transcription factor.

169

#### 170 ***crbzip1* mutants are hypersensitive to ER stress**

171 To characterize the function of *CrbZIP1* in *Chlamydomonas*, we used two independent lines  
172 of *crbzip1* mutants: *crbzip1-1*, which was isolated from mutant pools of *Chlamydomonas*  
173 strain C-9, and *crbzip1-2*, which was obtained from the *Chlamydomonas* mutant library  
174 (<https://www.chlamylibrary.org>), derived from the CC-4533 parental line. The *APHVIII*  
175 cassette, for selection on paromomycin, was inserted into the first exon of *CrbZIP1* in both  
176 mutants (Figure 2A). Immunoblot analysis showed that *crbzip1-1* and *crbzip1-2* did not  
177 contain a detectable level of CrbZIP1 proteins, indicating that both are *crbzip1* knockout  
178 mutants (Supplemental Figure 3B). We also generated complementation lines for the  
179 *crbzip1-1* and *crbzip1-2* mutants, named *crbzip1-1C* and *crbzip1-2C*, respectively, by





180 introducing *CrbZIP1* genomic DNA into each mutant background and confirmed that the  
 181 complementation lines expressed *CrbZIP1s* transcript and produced CrbZIP1 proteins  
 182 (Supplemental Figure 3).

183 In the absence of tunicamycin, there was no difference in growth among *crbzip1-1*,  
 184 *crbzip1-2*, their parental strains, and the complementation lines (Control in Figures 2B and  
 185 2C). However, after 48 h of treatment with 1  $\mu\text{g}/\text{mL}$  tunicamycin, the two *crbzip1* knockout  
 186 lines exhibited severe chlorosis, while their parental strains maintained green color (Figure  
 187 2B). Under ER stress conditions, growth was restored in the complementation lines to the  
 188 level of the parental lines (Figures 2B and 2C), indicating that the mutations in *CrbZIP1* in  
 189 the *crbzip1* mutants were responsible for their tunicamycin-sensitive phenotype. Whereas the  
 190 cell numbers of the parental strains and complemented strains were slightly increased after  
 191 48 h of tunicamycin treatment, those of the *crbzip1* mutants were unchanged (Figure 2C).  
 192 However, at 24 h of treatment, almost all *crbzip1* mutant cells were stained by propidium  
 193 iodide, a dye that permeates dead or damaged cells (Figure 2C), suggesting serious damage

194 or death of the cells.

195 Next, we observed the lipid droplets inside the cells using the lipid-specific fluorescent  
196 dye, Nile red. *crbzip1* mutants accumulated lipid droplets under ER stress, although parental  
197 strains and complemented strains barely displayed the Nile red fluorescence (Figure 2D). The  
198 lipid droplets in *crbzip1* mutants seemed to be present in extraplastidic regions (Figure 2D).

199

### 200 **CrbZIP1 is necessary for the expression of UPR genes under ER stress**

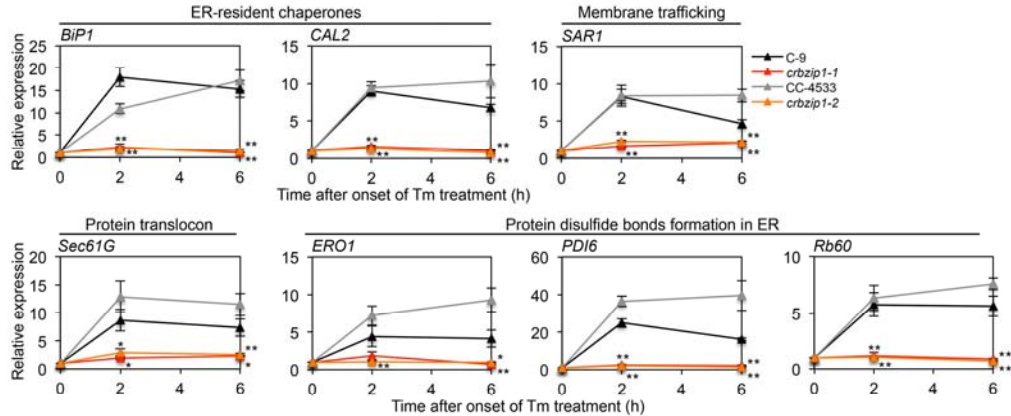
201 If CrbZIP1 has important functions in the ER stress response, the *crbzip1* mutants might  
202 differ from their parental lines in the activation of genes that confer ER stress resistance.  
203 Indeed, under tunicamycin treatment, the expression of genes encoding the chaperones BiP1  
204 and CAL2, membrane-trafficking protein SAR1, translocon SEC61G, and disulfide forming  
205 proteins ERO1, PDI6, and Pb60/PDI1A, which were reported to be involved in the ER stress  
206 response (Perez-Martin et al., 2014), was upregulated only in the parental lines, and not in the  
207 *crbzip1* mutants (Figure 3).

208 Interestingly, we found that the fold induction of expression by ER stress of  
209 *CrbZIP1s* and many UPR genes (*CAL2*, *SAR1*, *SEC61G*, *ERO1*, and *PDI6*) was higher in  
210 CC-4533 than in C-9 cells (Figure 3, Supplemental Figure 3A). This was because the  
211 expression levels of these genes were lower in CC-4533 cells than in C-9 cells at the 0-h time  
212 point (Supplemental Table 1). The difference in basal expression level of the  
213 ER-stress-related genes might be due to the large variations in genome sequences among  
214 individual *Chlamydomonas* strains (Siaut et al., 2011; Gallaher et al., 2015).

215

### 216 **Lipid remodeling is altered in the *crbzip1* mutants upon ER stress**

217 Lipid remodeling is known to be essential for cell survival under cold stress (Moellering et al.,  
218 2010), but it is not clear if it also plays a role in the cell's response to ER stress. To  
219 investigate the mechanisms underlying ER stress management, we analyzed the entire  
220 glycerolipid profiles of the *crbzip1* mutants and their parental strains under ER stress. After  
221 tunicamycin treatment, the mol% of TAG content did not increase very much from the  
222 starting value (0 h) in the parental lines; however, this value dramatically increased in the  
223 *crbzip1-1* and *crbzip1-2* mutants and was 5.8- and 9.4-fold that of each parental strain,  
224 respectively (Figure 4A, red boxes). Consistent with this result, the expression level of the  
225 gene encoding a key enzyme involved in TAG biosynthesis, ER-type DGTT1, was  
226 up-regulated only in the *crbzip1* mutants after tunicamycin treatment (Figures 4B and 4C),  
227 but the expression of genes encoding other acyltransferases was not up-regulated in the



**Figure 3. Expression levels of ER stress marker genes in *crbzip1* mutants and their parental strains.**

Cells were treated with 1  $\mu\text{g}/\text{mL}$  tunicamycin (Tm) for the indicated periods. The RT-qPCR results were normalized by the level of *RPL17* expression and the fold changes of expression levels at 2 h and 6 h were determined relative to the levels at 0 h. Error bars represent standard errors based on four biological replicates from two individual experiments. Statistical analysis was carried out using a Student's *t*-test (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ) and the Wilcoxon rank sum test (Supplemental Dataset 1).

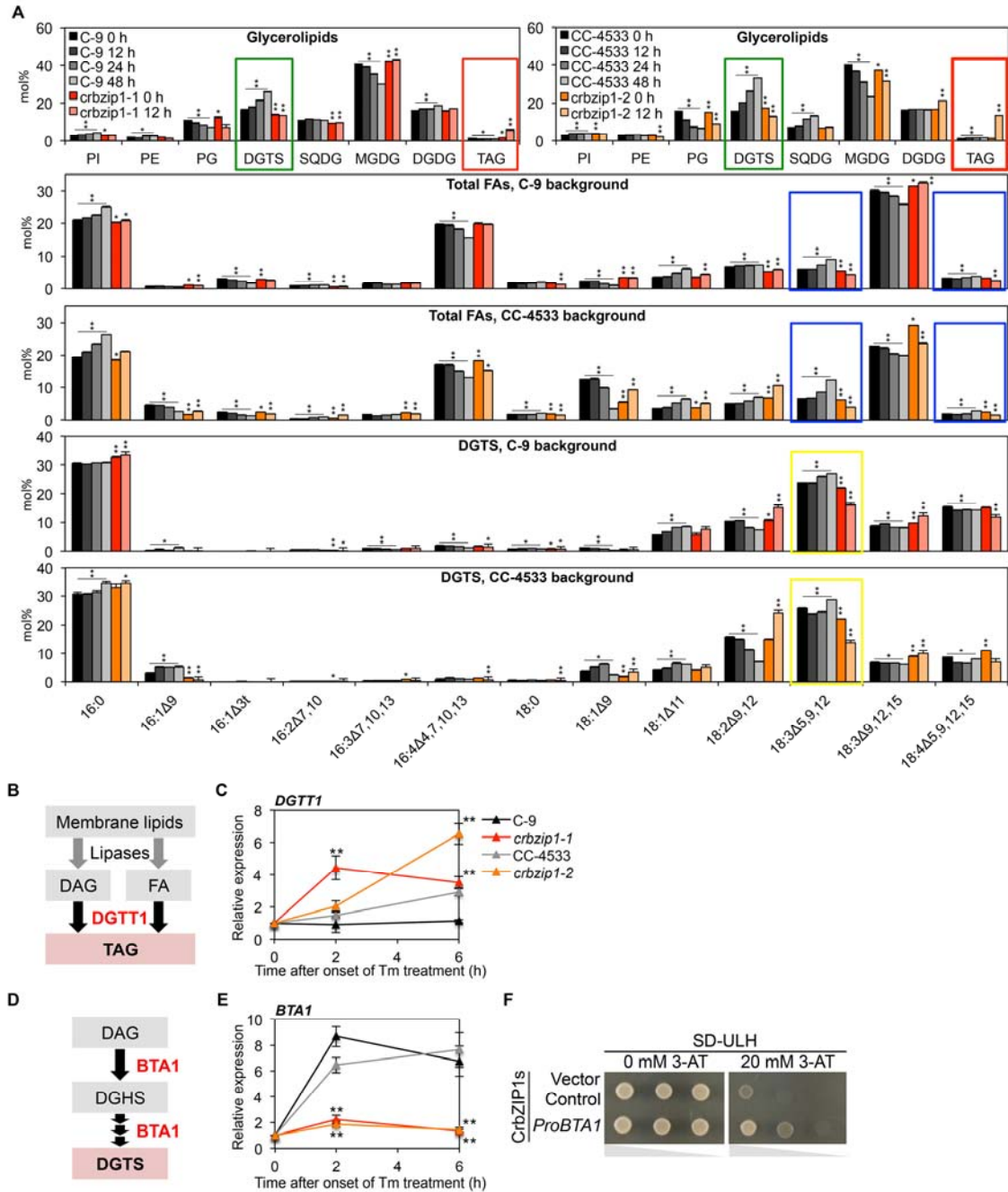
228 *crbzip1* mutants (Supplemental Figure 4).

229 To determine the source of the fatty acids used for TAG biosynthesis in the *crbzip1*  
 230 mutants, we compared the major glycerolipid content of *crbzip1* cells with that of their  
 231 parental lines. After 12 h of treatment, the most striking difference was the mol% of a major  
 232 extraplastidic membrane lipid, DGTS (Giroud et al., 1988), which decreased in *crbzip1*  
 233 mutants, but increased in parental lines (Figure 4A, green boxes). This result suggests that the  
 234 fatty acids in the TAGs formed in *crbzip1* cells during ER stress were derived from ER  
 235 membrane lipid. To determine how DGTS levels increased in the parental lines, we assayed  
 236 the expression level of *BTA1*, which encodes a key enzyme in DGTS biosynthesis in  
 237 *Chlamydomonas* (Riekhof et al., 2005). *BTA1* expression increased under ER stress, in the  
 238 parental lines and complementation lines, but not in the *crbzip1* mutants (Figures 4D and 4E,  
 239 Supplemental Figure 5). Furthermore, CrbZIP1s protein directly bound to the promoter  
 240 region of *BTA1* in a yeast one-hybrid assay (Figure 4F). These results suggest that CrbZIP1s  
 241 up-regulates *BTA1* expression and thus the production of DGTS synthesis under ER stress in  
 242 *Chlamydomonas*.

243

244 **CrbZIP1 expression is necessary for ER stress-induced biosynthesis of pinolenic acid,**  
 245 **which enhances ER stress tolerance**

246 Next, we analyzed the fatty acid composition of the *crbzip1* mutants and their parental strains.  
 247 During ER stress, the abundance of pinolenic acid (18:3 $\Delta$ 5,9,12) and coniferonic acid  
 248 (18:4 $\Delta$ 5,9,12,15) in the total fatty acids was increased in the parental strains but was reduced  
 249 in the *crbzip1* mutants (Figure 4A, blue boxes). Subsequent analysis of fatty acid composition  
 250 of individual glycerolipids revealed that the pinolenic acid content in DGTS differed



**Figure 4. ER-stress-induced lipid remodeling differs between the *crbzip1* mutant and their parental lines.**

251 dramatically between the *crbzip1* mutants and their parental lines (Figure 4A, Supplemental  
 252 Figure 6). In DGTS, the pinolenic acid content slightly increased in parental strains but  
 253 decreased greatly in the *crbzip1* mutants after tunicamycin treatment (Figure 4A, yellow  
 254 boxes). Pinolenic acid biosynthesis in the ER requires the desaturases FAD2 and CrDES in  
 255 *Chlamydomonas* (Kajikawa et al., 2006). FAD2 and CrDES expression increased  
 256 significantly in the parental strains, but not in the *crbzip1* mutants, upon treatment with  
 257 tunicamycin (Figure 5B), suggesting that CrbZIP1 induces these desaturases. This possibility

**Figure 4. ER-stress-induced lipid remodeling differs between the *crbzip1* mutant and their parental lines.**

(A) Levels of fatty acids (FAs) in glycerolipids and the fatty acid composition of total fatty acids and of diacylglyceryltrimethylhomoser (DGTS). Lipids extracted from *crbzip1* mutants and their parental strains treated with 1 µg/mL tunicamycin (Tm) for the indicated periods were analyzed using GC-FID. Error bars represent standard errors based on three biological replicates. Red box indicates TAG accumulation in *crbzip1* cells. Green box represents the induction of DGTS only in the parental strains. Blue boxes indicate the decreases of pinolenic acid in total FAs in *crbzip1* cells. Yellow boxes indicate the dramatic reductions of pinolenic acid in DGTS, phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), sulfoquinovosyldiacylglycerol (SQDG), monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and triacylglycerol (TAG). Statistical analysis was carried out using a Student's *t*-test (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ , Supplemental Dataset 2), and the significant differences between the mutant and the parental strain at the same time point are marked.

(B) Major TAG biosynthesis pathway mediated by DGTT1 in *Chlamydomonas*. DGTT1 is a critical enzyme for TAG synthesis in *Chlamydomonas*.

(C) Expression levels of *DGTT1* under ER stress. Cells were treated with 1 µg/mL Tm for the indicated periods. The RT-qPCR results were normalized by the level of *RPL17* expression and the fold changes of expression levels were determined relative to the levels at 0 h. Error bars represent standard errors based on four biological replicates at 0, 2, and 6 h. Statistical analysis was carried out using a Student's *t*-test (\*\*,  $p < 0.01$ ) and the Wilcoxon rank sum test (Supplemental Dataset 1).

(D) DGTS biosynthesis pathway mediated by BTA1 in *Chlamydomonas*. BTA1 is a critical enzyme for DGTS biosynthesis in *Chlamydomonas*.

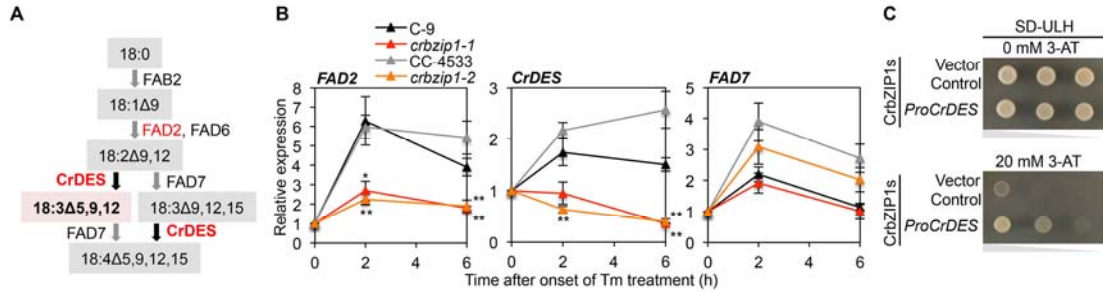
(E) Expression level of *BTA1* in *Chlamydomonas* cells subjected to ER stress. Experimental conditions and data processing were the same as in (C).

(F) Yeast one-hybrid assay showing that CrbZIP1s interacts directly with the promoter region of *BTA1*. Yeast cells were spotted on medium containing SD-Leu-Ura-His with or without 3-AT with a series of 1:10 dilutions.

258 was supported by a yeast one-hybrid assay, which revealed that CrbZIP1s interacted directly  
259 with the promoter region of *CrDES* (Figure 5C). By contrast, the *FAD7* transcript level was  
260 induced both in the parental lines and in the *crbzip1* mutants (Figure 5B), indicating that the  
261 production of alpha-linolenic acid (18:3Δ<sup>9,12,15</sup>) or coniferonic acid (18:4Δ<sup>5,9,12,15</sup>) is not  
262 the downstream target of CrbZIP1.

263 We then hypothesized that the induction of pinolenic acid in *Chlamydomonas* cells  
264 might protect the cells from ER stress. To address this possibility, we isolated *crdes1-1* and  
265 *crdes1-2* mutants in the C-9 and CC-4533 background, respectively (Figure 6A,  
266 Supplemental Figure 7). The *crdes1-1* mutant did not contain detectable amounts of pinolenic  
267 acid (18:3Δ<sup>5,9,12</sup>) or coniferonic acid (18:4 Δ<sup>5,9,12,15</sup>; Figure 6B, blue arrows in Total FAs),  
268 and the *crdes1-2* mutant contained lower levels of the two compounds than the parental line  
269 (Figure 6B, green arrows in Total FAs, yellow boxes in DGTS; Supplemental Figure 8,  
270 yellow boxes in PE and TAG), suggesting that *crdes1-1* is a knockout mutant and *crdes1-2* is  
271 a knockdown mutant. The fatty acid composition of other major glycerolipid species was not  
272 altered much in the *crdes* mutants (Supplemental Figure 8). Although the pinolenic acid  
273 content of PE and DGTS was reduced relative to the parental lines, the DGTS and PE  
274 contents were maintained in the *crdes* mutants (Figure 6B, Glycerolipids). The increase in  
275 alpha-linolenic acid (18:3Δ<sup>9,12,15</sup>) appeared to compensate for the loss of pinolenic acid in  
276 PE and DGTS in both the *crdes* mutants (Figures 6B, Supplemental Figure 8, red boxes).

277 Next, we investigated whether the two *crdes* mutants differed from their parental  
278 lines with respect to ER stress resistance. No difference in growth between any of the  
279 *Chlamydomonas* strains was apparent when the cells were cultured on normal TAP medium  
280 (Figure 6C, TAP). However, when the strains were spotted on TAP medium containing 0.2



**Figure 5. CrbZIP1s promotes the biosynthesis of pinolenic acid (18:3Δ5,9,12) under ER stress.**

(A) C18 FA biosynthesis pathway in *Chlamydomonas*.

(B) Relative transcript abundance of desaturase genes after tunicamycin (Tm) treatment. Cells were treated with 1 μg/mL Tm for the indicated periods. The RT-qPCR results were normalized by the level of *RPL17* expression and the fold changes of expression levels were determined relative to the levels at 0 h. Error bars represent standard errors based on four biological replicates for 0, 2, and 6 h. Statistical analysis was carried out using a Student's *t*-test (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ) and the Wilcoxon rank sum test (Supplemental Dataset 1).

(C) Yeast one-hybrid assay showing that CrbZIP1s interacts directly with the promoter region of *CrDES*. Yeast cells were spotted on medium containing SD-Leu-Ura-His with or without 3-AT with a series of 1:10 dilutions.

281 μg/mL tunicamycin, *crdes* mutants exhibited hypersensitivity to ER stress, similarly to  
 282 *crbzip1* mutants (Figure 6C, TAP + Tm). These data suggest that CrbZIP1-mediated pinolenic  
 283 acid biosynthesis improves the survival of *Chlamydomonas* exposed to ER stress.

284

285

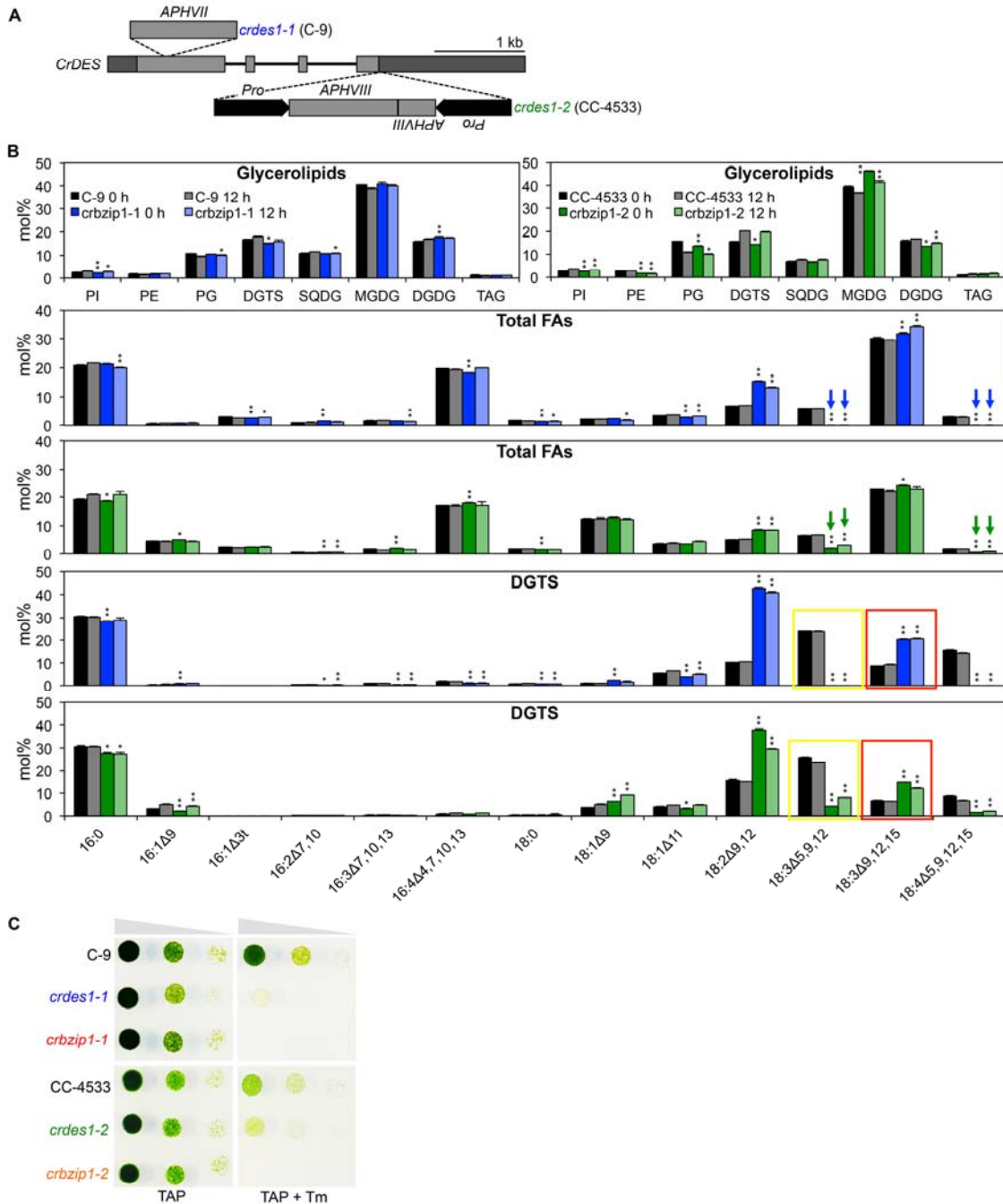


Figure 6. *crdes* knockdown mutants are hypersensitive to the ER stress imposed by tunicamycin treatment.

286 **DISCUSSION**

287 **CrbZIP1 functions downstream of CrIRE1 and plays a critical role in the ER stress**  
 288 **response**

289 In microalgae, no transcription factor has been identified that functions downstream of the  
 290 ER stress sensor. In this study, we established that the *Chlamydomonas* transcription factor  
 291 CrbZIP1 functions downstream of CrIRE1, and activates the expression of many genes that

**Figure 6. *crdes* knockdown mutants are hypersensitive to the ER stress imposed by tunicamycin treatment.**

(A) Schematic representation of insertion sites of the *APHVII* and *APHVIII* cassettes in the genomic sequence of *CrDES* in *crdes* mutants. The positions of UTRs, exons, and introns are indicated by dark-gray boxes, gray boxes, and lines, respectively.

(B) Levels of fatty acids (FAs) in glycerolipids and fatty acid composition of total fatty acids, phosphatidylethanolamine (PE), and diacylglyceryltrimethylhomo-Ser (DGTS). Total lipids were extracted from cells treated with 1  $\mu$ g/mL tunicamycin (Tm) for the indicated periods. Blue arrows and green arrows indicate absence or reduction of pinolenic acid and coniferonic acid in *crdes1-1* and *crdes1-2* mutants, respectively. Yellow boxes indicate absence or reduction of pinolenic acid in *crdes1-1* or *crdes1-2* mutants, respectively. Red boxes indicate compensatory increases of alpha-linolenic acid in *crdes1* mutants. Error bars represent standard errors based on three biological replicates. Statistical analysis was performed as described in Supplemental Dataset 3.

(C) Growth of the indicated *Chlamydomonas* cell lines spotted on TAP medium supplemented with or without 0.2  $\mu$ g/mL Tm with a series of 1:10 dilutions.

292 contribute to survival under ER stress. The following observations support this conclusion.  
293 First, *CrbZIP1* mRNA was spliced under ER stress in *Chlamydomonas* cells, but this splicing  
294 activity was very low in *crire1* knockdown mutants (Figure 1B). Second, two independent  
295 mutant alleles of *CrbZIP1*, *crbzip1-1* and *crbzip1-2*, which did not exhibit any growth defect  
296 under normal conditions, exhibited increased sensitivity to ER stress: they were completely  
297 bleached within 2 days of exposure to a concentration of tunicamycin that did not induce  
298 chlorosis in their parental lines (Figure 2B). Third, *CrbZIP1* upregulated the expression of ER  
299 stress response genes, including chaperone genes and lipid biosynthesis genes (Figure 3). We  
300 also obtained data that are consistent with *CrbZIP1* having a role in the ER stress response:  
301 *CrbZIP1* levels increased under ER stress (Figure 1E), and the *CrbZIP1*s protein encoded by  
302 the spliced form of *CrbZIP1* was localized in the nucleus (Figure 1F).

303

304 **Increase in pinolenic acid content protects the cells during ER stress**

305 Pinolenic acid levels increased in the parental strains, but dramatically decreased in the  
306 *crbzip1* mutants under ER stress (Figure 4A). Pinolenic acid (18:3 $\Delta$ 5,9,12), a  $\Delta$ 5-desaturated  
307 metabolite of linoleic acid (18:2 $\Delta$ 9,12), is found in the oil of Korean pine (*Pinus koraiensis*)  
308 nuts and needles and in some microalgae. A polymethylene group within the carbon skeleton  
309 of pinolenic acid makes it structurally distinct from its positional isomer gamma-linolenic  
310 acid (18:3 $\Delta$ 6,9,12). Pinolenic acid was demonstrated to provide specific health benefits not  
311 imparted by several other 18:3 fatty acid species (Asset et al., 2000; Asset et al., 2001; Lee et  
312 al., 2004; Pisman et al 2008). *Chlamydomonas* was reported to produce pinolenic acid, but  
313 the physiological role of this fatty acid in this alga remained unknown (Giroud et al., 1988).  
314 In this study, we isolated two independent mutants of *CrDES*, a desaturase required for  
315 pinolenic acid production, and found that the *crdes* mutants were much more impaired in  
316 growth in medium containing tunicamycin than were their parental lines (Figure 6C). Notably,  
317 the *crdes1-1* knockout mutant displayed ER stress hypersensitivity comparable to that of the  
318 *crbzip1-1* mutant (Figure 6C), suggesting that the upregulation of *CrDES* is one of the major  
319 routes through which *CrbZIP1* confers ER stress resistance in *Chlamydomonas*. Furthermore,



320 the yeast one-hybrid assay suggested that CrbZIP1s directly regulates *CrDES* expression  
321 (Figure 5C). Therefore, CrbZIP1-dependent pinolenic acid biosynthesis seems to be  
322 necessary for effective protection of this microalga from ER stress. Since pinolenic acid is  
323 produced only in a limited number of organisms, these results also indicate that ER stress  
324 tolerance mechanisms differ among various organisms.

325         How does pinolenic acid enhance tolerance to ER stress in *Chlamydomonas*?  
326 Previous reports revealed that reactive oxygen species (ROS) are induced by ER stress in  
327 *Chlamydomonas* cells (Pérez-Martín et al., 2014), and that polyunsaturated fatty acids in  
328 membrane lipids act as sinks for ROS in plastids (Mene-Saffrane et al., 2009; Zeoeller et al.,  
329 2012). Thus, pinolenic acid might be able to mitigate ROS toxicity. However, this general  
330 effect of polyunsaturated fatty acids does not explain the effect of pinolenic acid in ER stress  
331 resistance in *Chlamydomonas*, since the levels of another polyunsaturated fatty acid,  
332 alpha-linolenic acid (18:3 $\Delta$ 6,9,12), increased in DGTS and PE in *crdes* mutants (Figure 6B,  
333 Supplemental Figure 8), but failed to protect the cells from ER stress (Figure 6C). Another  
334 possibility is that pinolenic acid functions as a signaling molecule, much like jasmonic acid in  
335 many terrestrial plants. Trees in the Pinus family (Pinaceae) are among the longest living  
336 plants on Earth (Rogers and Clifford, 2006; Withington et al., 2006). Thus, it is tempting to  
337 speculate that pinolenic acid in the leaves of these trees confers ER stress resistance and  
338 consequently contributes to the longevity of the plant. Further studies are needed to  
339 understand the protective function of pinolenic acid in *Chlamydomonas* subjected to ER  
340 stress, and it would be interesting to test whether pinolenic acid confers ER stress resistance  
341 in other organisms, too.

342

#### 343 **Increase of DGTS under ER stress**

344 Some algae, including *Chlamydomonas*, have been reported to contain DGTS but not  
345 phosphatidylcholine (PC) in the ER membrane (Kato et al., 1996). Our study revealed that  
346 CrbZIP1 directly upregulated the expression of the DGTS synthase gene *BTAL*, and that  
347 DGTS content significantly increased in the parental lines but not in the *crbz1pl* mutants  
348 under ER stress (Figure 4A, Glycerolipids). An increase in ER membrane lipids was also  
349 observed in other organisms exposed to ER stress. In animals, XBP1 plays an important role  
350 in the biosynthesis of PC, the main ER phospholipid, which allows ER membrane expansion  
351 under ER stress (Sriburi et al., 2007). Furthermore, in yeast, the increase in ER size by  
352 upregulation of PC biosynthesis improves ER stress tolerance (Schuck et al., 2009). By  
353 analogy with these studies in animals and yeast, we hypothesized that the increase in DGTS

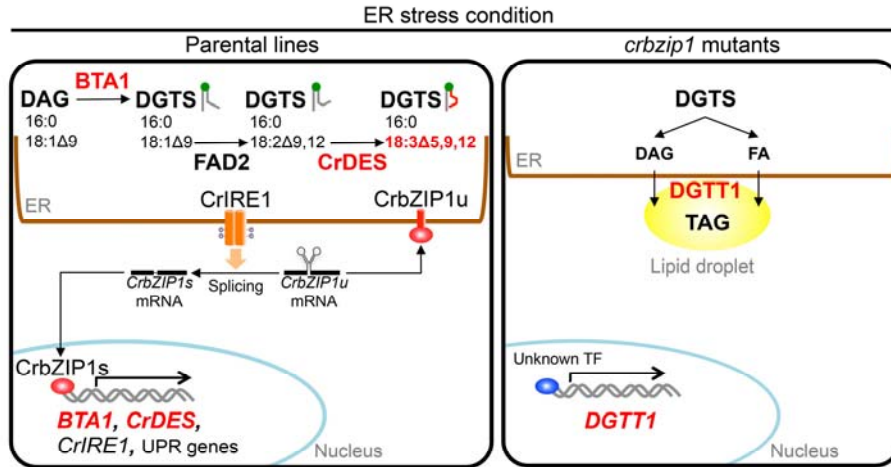
354 levels in *Chlamydomonas* cells might contribute to their survival under ER stress by  
355 expanding the ER membrane. In DGTS, the polar betaine trimethylglycine is linked by an  
356 ether bond at the *sn*-3 position of the glycerol moiety. Trimethylglycine is an osmolyte that  
357 stabilizes proteins under many stress conditions (Auton et al., 2011). Covering the surface of  
358 the ER lumen with trimethylglycine might mediate protein folding under ER stress in  
359 *Chlamydomonas*. Thus, it would be interesting to study the biological function of this betaine  
360 lipid further.

361

### 362 ***crbzip1* mutations induce hypersensitivity to ER stress and promote the formation of** 363 **lipid droplets**

364 *crbzip1* mutants accumulated lipid droplets under the ER stress induced by tunicamycin  
365 (Figure 2D). Lipid droplets were also observed in *crire1* mutants treated with the same  
366 concentration (1  $\mu$ g/mL) of tunicamycin (Yamaoka et al., 2018). These results imply that  
367 mutations in the CrIRE1/CrbZIP1 pathway disrupt the regular UPR under ER stress, resulting  
368 in the accumulation of lipid droplets and rendering these mutants more vulnerable to stress  
369 than their parental lines. The biosynthesis of the lipids accumulated in the lipid droplets  
370 seemed to be catalyzed by an acyltransferase, *DGTT1*, previously shown to play a major role  
371 in TAG accumulation under nitrogen starvation stress (Boyle et al., 2012), since the gene  
372 encoding this protein was expressed at higher levels in *crbzip1* mutants than in their parental  
373 strains under ER stress (Figure 4C).

374 In summary, we established CrbZIP1 as the target of CrIRE1 under ER stress, and  
375 provided insight into the CrIRE1/CrbZIP1-mediated response to ER stress in  
376 *Chlamydomonas* (Figure 7). Under the ER stress induced by 1  $\mu$ g/mL tunicamycin, *CrbZIP1*  
377 is activated by cleavage and upregulates the expression of general UPR genes and of genes  
378 encoding DGTS synthase and pinolenic acid synthase, and thereby overcomes the stress.  
379 However, in the absence of functional CrIRE1/CrbZIP1 proteins, *Chlamydomonas* cells  
380 exhibited another stress response strategy and stress symptom, namely the induction of  
381 acyltransferase and the formation of lipid droplets. Our data clearly indicate that lipid  
382 modifications contribute to the survival of the *Chlamydomonas* cells exposed to ER stress. It  
383 would be very interesting to test whether similar lipid modifications occur during the ER  
384 stress response in other organisms. We predict that some aspects of the lipid modification  
385 revealed here might be common to the ER stress response in many organisms, since the  
386 IRE1-mediated ER stress response pathway is highly conserved (Howell 2013; Ron and  
387 Walter 2007; Yamaoka et al., 2018).



**Figure 7. Proposed model for a mechanism of ER stress responses in *crbzip1* mutants and their parental lines in *Chlamydomonas*.**

Left: In the parental lines, activated CrIRE1 splices *CrbZIP1u* mRNA to produce *CrbZIP1s*, which is translated to CrbZIP1s. CrbZIP1s enters the nucleus, where it induces the expression of UPR genes and ER membrane lipid biosynthesis genes, such as *BTA1* and *CrDES*. The dimerization and phosphorylation of CrIRE1 are drawn based on reports in other organisms but have not been revealed in *Chlamydomonas*.

Right: The *crbzip1* mutation upregulates TAG biosynthesis from the fatty acids hydrolyzed from a major ER membrane lipid, DGTS, resulting in the formation of lipid droplets, which store TAGs.

388

389

## 390 **METHODS**

### 391 **Strains and culture conditions**

392 The *Chlamydomonas* strain C-9 (CC-5098, *mt<sup>-</sup>*), originally provided by the IAM Culture  
393 Collection at The University of Tokyo and available from the Chlamydomonas Resource  
394 Center, was used as the parental strain. The *crbzip1-1* and *crdes1-1* mutants, containing the  
395 *APHVIII* and *APHVII* insertions, respectively, were isolated in accordance with a previous  
396 report (Yamano et al., 2015). The *Chlamydomonas* strain CC-4533 (*cw15 mt<sup>-</sup>*) and the  
397 *crbzip1-2* (LMJ.RY0402.143080) and *crdes1-2* (LMJ.RY0402.087433) mutants were  
398 obtained from the Chlamydomonas Genetics Center (USA) (Li et al., 2016). *Chlamydomonas*  
399 cells were cultured in an Erlenmeyer flask containing Tris-acetate-phosphate (TAP) medium  
400 (Harris, 2009) at 23°C under continuous light ( $75 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) supplied by tubular  
401 fluorescent lamps (color temperature 4000K) with shaking at 190 rpm. Cells in the exponential  
402 growth phase ( $3 \times 10^6$  cells/mL) were treated with the indicated concentration of tunicamycin  
403 (Sigma-Aldrich, T7765) diluted from a 5 mg/mL stock in 0.05 N NaOH or DTT  
404 (dithiothreitol; Duchefa Biochemie, Netherlands) diluted from a 1 M stock in water. Dead  
405 cells were stained with 2  $\mu\text{g/mL}$  propidium iodide for 15 min in darkness, as described in Kim  
406 et al. (2015), and cells immobilized in 1% (w/v) low-gelling agarose (Sigma-Aldrich) were  
407 counted using a hemocytometer under a fluorescence microscope (Nikon Optiphot  
408 microscope) with a TRITC filter.

409

### 410 **Phylogenetic tree analyses**

411 Amino acid sequences were obtained from Phytozome v12.1 (<https://phytozome.jgi.doe.gov>),  
412 TAIR (<https://www.arabidopsis.org>), and the *Saccharomyces* Genome Database  
413 (<http://www.yeastgenome.org>) and were aligned using the Clustal Omega algorithm (Sievers  
414 et al., 2011). The phylogenetic tree was constructed using MEGA 7.0 (Kumar et al., 2016) with  
415 1,000 bootstrap replicates.

416

### 417 **Immunoblot analysis**

418 Anti-CrbZIP1 antibody was generated against a synthetic peptide containing the 23-amino  
419 acid fragment located at the N-terminus of CrbZIP1 (GPSLLDDELFSFDLMQFLETIEG).  
420 Rabbits were injected with the peptide for the production of anti-CrbZIP1 polyclonal  
421 antibody (Young In Frontier, South Korea). To detect CrbZIP1 protein levels, total protein  
422 was extracted from *Chlamydomonas* cells and 40  $\mu\text{g}$  of total protein was subjected to  
423 immunoblot analysis as described previously (Song et al., 2004).

424

425 **Subcellular localization of CrbZIP1 in *Nicotiana benthamiana* epidermal cells**

426 *CrbZIP1u* and *CrbZIP1s* cDNA were introduced into pGWB5 (Nakagawa et al., 2007).

427 *Arabidopsis* BiP1 cDNA were introduced into pGWB560 (Nakagawa et al., 2008). *N.*

428 *benthamiana* leaves were infiltrated as described previously (Sparkes et al., 2006).

429

430 **Microscopy analysis**

431 To visualize lipid droplets, cells were stained with 1 µg/mL Nile red (Sigma-Aldrich) diluted

432 from a 0.1 mg/mL stock solution in acetone for 20 min in darkness at room temperature, and

433 the cells were immobilized in 1% (w/v) low-gelling agarose (Sigma-Aldrich). To visualize the

434 nucleus, the DAPI staining was performed as described by Yamaoka et al. (2011).

435 Fluorescence was observed using a confocal laser-scanning microscope (Olympus).

436

437 **RNA extraction and quantification**

438 Total RNA was extracted and quantified as described previously (Jang et al., 2015), with a few

439 modifications. Total RNA was isolated using homemade TRIzol, and cDNA was synthesized

440 using a RevertAid First Strand cDNA Synthesis Kit (K1621, Thermo Scientific) and 3 µg of

441 total RNA. Quantitative RT-PCR was performed using SYBR Premix Ex Taq (Takara) and the

442 primer sets are listed in Supplemental Table 1. The quantitative RT-PCR results were

443 normalized by the level of *RPL17* expression (Yamaoka et al., 2018).

444

445 **Isolation of complementation strains**

446 The promoter region of the *Chlamydomonas* expression vector pChlamy\_1 (Thermo

447 Scientific) was removed, and the *CrbZIP1* promoter region fused with the 5'-UTR (2135 bp)

448 and the *CrbZIP1* genomic DNA with the 3'-UTR (2911 bp) was inserted as a *Xba*I fragment

449 (pChlamy-CrbZIP1). *crbizp1-1* and *crbizp1-2* were transformed with pChlamy-CrbZIP1,

450 and the transformants were selected by hygromycin tolerance. CrbZIP1 proteins from 96

451 transformants were individually analyzed by immunoblot assay, and the transformants that

452 contained similar amounts of CrbZIP1 proteins to the parental strains were selected as

453 complementation lines of *crbizp1* mutants.

454

455 **Lipid extraction and quantification**

456 Lipids were extracted and analyzed as described previously (Kim et al., 2013) with

457 modifications. Briefly, 40 mL of *Chlamydomonas* cell culture (approximately  $5 \times 10^6$  cells/mL)  
458 was harvested by centrifugation at 2,000 g for 5 min at room temperature and used for total  
459 lipid extraction. To quantify TAGs, 1 mg of the total lipid extract was separated on a thin-layer  
460 chromatography (TLC) plate using the following solvent mixture: hexane:diethyl ether:acetic  
461 acid (80:30:1, by volume). To separate membrane lipids, 1 mg of the total lipid extract was  
462 separated on a TLC plate using the following solvent mixture: chloroform:methanol:acetic  
463 acid:water (75:13:9:3, by volume). Lipid spots were visualized under UV after spraying with  
464 0.01% (w/v) primuline (Sigma) dissolved in acetone:H<sub>2</sub>O (4:1, v/v). Lipid bands were  
465 recovered from the plate and quantified by a gas chromatograph (GC-FID, SHIMADZU  
466 GC-2010) equipped with an HP-INNOWax capillary column (30 m × 0.25 mm, 0.25 μm film  
467 thickness, Agilent Technologies) after transesterification.

468

#### 469 **Yeast one-hybrid assay**

470 Yeast one-hybrid assays were carried out using the Matchmaker™ Yeast One-HybridSystem  
471 (Clontech, USA), as described in the manufacturer's protocol. The promoters of *BTA1* (655 bp)  
472 and *CrDES* (736 bp) were cloned into the pHISi vector, and codon-optimized CrbZIP1s for  
473 yeast expression was subcloned into the pGAD424 vector using the primers listed in Table S1.  
474 Yeast cells were spotted on medium containing SD-Leu-Ura-His with or without 3-AT with a  
475 series of 1:10 dilutions.

476

#### 477 **Accession Numbers**

478 *Chlamydomonas* genes analyzed in this study were identified in Phytozome v12  
479 (<http://phytozome.jgi.doe.gov/pz/portal.html>) under the following accession numbers:

480 *CrbZIP1*, Cre05.g238250.t1.2; *CrIRE1*, Cre08.g371052.t1.1; *BiP1*, Cre02.g080700.t1.2;  
481 *CAL2*, Cre01.g038400.t1.2; *SAR1*, Cre11.g468300.t1.2; *SEC61*, Cre16.g680230.t1.1; *ERO1*,  
482 Cre17.g723150.t1.3; *PDI6*, Cre12.g518200.t1.3; *Rb60*, Cre02.g088200.t1.2; *DGTT1*;  
483 Cre12.g557750.t1.1; *BTA1*, Cre07.g324200.t1.2; *FAD2*, Cre17.g711150.t1.1; *CrDES*,  
484 Cre10.g453600.t2.1; *FAD7*, Cre01.g038600.t1.2; *PDAT*, Cre02.g106400.t1.1; *DGAT*,  
485 Cre01.g045903.t1.1; *DGTT2*, Cre09.g386912.t1.1; *DGTT3*, Cre06.g299050.t1.2; *DGTT4*,  
486 Cre03.g205050.t1.2. Arabidopsis genes analyzed in this study were identified in TAIR  
487 (<https://www.arabidopsis.org>) under the following accession numbers: *AtbZIP60*,  
488 AT1G42990.1; *AtBiP1*, AT5G28540.1. The yeast gene analyzed in this study was identified in  
489 the *Saccharomyces* Genome Database (<http://www.yeastgenome.org>) under the following  
490 accession number: *Hac1*, S000001863.

491

492 **Supplemental Data**

493 **Supplemental Figure 1. The splicing motif of *CrbZIP1* mRNA differs from that of**  
494 ***AtbZIP60*, *ScHAC1*, and *HsXBPI* mRNA.**

495

496 **Supplemental Figure 2. Immunoblot analysis of CrbZIP1u and CrbZIP1s.**

497

498 **Supplemental Figure 3. Isolation of complementation strains of *crbzip1* mutants.**

499

500 **Supplemental Figure 4. Expression levels of acyltransferase genes in *crbzip1* mutants and**  
501 **their parental strains under the ER stress.**

502

503 **Supplemental Figure 5. Expression levels of *BTA1* and *CrDES* were recovered in the**  
504 ***crbzip1-1C* and *crbzip1-2C* lines.**

505

506 **Supplemental Figure 6. Fatty acid compositions of glycerolipids in *crbzip1* mutants**  
507 **subjected to ER stress.**

508

509 **Supplemental Figure 7. Isolation of *Chlamydomonas crdes1-1* and *crdes1-2* mutants.**

510

511 **Supplemental Figure 8. Fatty acid compositions of glycerolipids in *crdes* mutants**  
512 **subjected to ER stress.**

513

514 **Supplemental Table 1. Expression levels of ER stress sensor and ER stress marker genes**  
515 **in *crbzip1* mutants and their parental strains.**

516

517 **Supplemental Table 2. Primer sequences.**

518

519 **Supplemental Dataset 1. Statistical analyses for transcript levels.**

520

521 **Supplemental Dataset 2. Statistical analyses for lipid content of *crbzip1* mutants and**  
522 **their parental strains.**

523

524 **Supplemental Dataset 3. Statistical analyses for lipid content of *crdes* mutants and their**

525 **parental strains.**

526

## 527 **ACKNOWLEDGMENTS**

528 This work was supported by the Advanced Biomass R&D Center (ABC) of Global Frontier  
529 Project funded by the Ministry of Science, ICT and Future Planning  
530 (ABC-2015M3A6A2065746) awarded to Y. Lee, the Japan Society for the Promotion of  
531 Science KAKENHI (16H04805) and the Japan Science and Technology Agency, Advanced  
532 Low Carbon Technology Research and Development Program (ALCA, JPMJAL1105)  
533 awarded to H. Fukuzawa, Agence Nationale de la Recherche (ANR-MUSCA) awarded to Y.  
534 Li-Beisson, and the Korea Research Fellowship Program funded by the Ministry of Science,  
535 ICT and Future Planning through the National Research Foundation of Korea  
536 (2016H1D3A1909463) to Y. Yamaoka. We thank the *Chlamydomonas* Mutant Library Group  
537 at the Carnegie Institution for Science and the *Chlamydomonas* Resource Center at the  
538 University of Minnesota for providing the indexed *Chlamydomonas* insertional mutants.

539

## 540 **AUTHOR CONTRIBUTIONS**

541 Y.Y., Y.L.-B., and Y.L. contributed to design of the research. Y.Y., S.S., B.Y.C., H.K., and S.J.  
542 performed the research. T.Y. and H.F. produced mutant library using *Chlamydomonas* strain  
543 C-9. Y.Y. and S.S. isolated *crbzip1-1* mutant, and M.K. isolated *crdes1-1* mutant. Y.Y., F.K.,  
544 B.L., and Y.L.-B. performed lipid analysis. Y.Y., Y.L.-B., and Y.L. wrote and revised the  
545 manuscript.

546

## 547 **SUPPLEMENTAL DATA**

548 **Supplemental Figure 1. The splicing motif of *CrbZIP1* mRNA differs from that of**  
549 ***AtbZIP60*, *SchHAC1*, and *HsXBPI* mRNA.** Supports Figure 1C.

550 Stem-loop structures predicted for the mRNAs of *CrbZIP1*, *AtbZIP60*, *SchHAC1*, and *HsXBPI*.  
551 The conserved nucleotides essential for splicing of *AtbZIP60*, *SchHAC1*, and *HsXBPI* mRNAs  
552 are enclosed in boxes. Red arrowheads indicate the splicing sites observed in target mRNAs of  
553 IRE1. The structure of *CrbZIP1* was predicted using CentroidFold. The structures of *AtbZIP60*,  
554 *SchHAC1*, and *HsXBPI* were adapted from Nagashima et al., 2011.

555

556 **Supplemental Figure 2. Immunoblot analysis of *CrbZIP1u* and *CrbZIP1s*.** Supports  
557 Figure 1E.

558 (A) Immunoblot analysis of *CrbZIP1u* (53 kDa) and *CrbZIP1s* (33 kDa) using anti-*CrbZIP1*



559 antibody. Protein preparations from cells treated with 1  $\mu\text{g}/\text{mL}$  tunicamycin (Tm) or 5 mM  
560 dithiothreitol (DTT) for the indicated periods were subjected to immunoblot analysis.  
561 Coomassie brilliant blue staining (CBB) was used as a loading control.

562 (B) The size verification of CrbZIP1s expressed in *E. coli*. MBP-CrbZIPs was eluted from the  
563 crude protein preparation of *E. coli* containing pMAL-c2X-CrbZIP1s using amylose resin.  
564 MBP-CrbZIP1s was digested with the factor Xa, separated by SDS-PAGE, and then  
565 visualized by silver staining. Although the protein size of CrbZIP1s was predicted to be 33 kDa,  
566 CrbZIP1s from *Chlamydomonas* cells and recombinant CrbZIP1s protein expressed in *E. coli*  
567 were found at around 25 kDa, due to some unknown reason.

568

569 **Supplemental Figure 3. Isolation of complementation strains of *crbzip1* mutants.** Supports  
570 Figures 2A and 2B.

571 (A) Expression levels of CrbZIP1s in *crbzip1* mutants, their parental strains, and their  
572 complement strains. Cells were treated with 1  $\mu\text{g}/\text{mL}$  tunicamycin (Tm) for the indicated  
573 periods. The RT-qPCR results were normalized by the level of *RPL17* expression, and the fold  
574 changes of expression levels at 2 and 6 h were determined relative to the levels at 0 h. Error  
575 bars represent standard errors based on four biological replicates from two individual  
576 experiments. Statistical analysis was carried out using Student's *t*-test (\*,  $p < 0.05$ ; \*\*,  $p <$   
577 0.01) and the Wilcoxon rank sum test (Supplemental Dataset 1).

578 (B) Immunoblot analysis of CrbZIP1 proteins using anti-CrbZIP1 antibody. Protein  
579 preparations from cell lines treated with 1  $\mu\text{g}/\text{mL}$  tunicamycin (Tm) or 5 mM dithiothreitol  
580 (DTT) for 6 h were subjected to immunoblot analysis. Coomassie brilliant blue staining (CBB)  
581 was used as a loading control.

582

583 **Supplemental Figure 4. Expression levels of acyltransferase genes in *crbzip1* mutants and  
584 their parental strains under the ER stress.** Supports Figure 4C.

585 Cells were treated with 1  $\mu\text{g}/\text{mL}$  tunicamycin (Tm) for the indicated periods. The RT-qPCR  
586 results were normalized by the level of *RPL17* expression and the fold changes of expression  
587 levels at 2 h and 6 h were determined relative to the levels at 0 h. Error bars represent standard  
588 errors based on four biological replicates from two individual experiments. Statistical analysis  
589 was carried out using Student's *t*-test (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ) and the Wilcoxon rank sum test  
590 (Supplemental Dataset 1).

591

592 **Supplemental Figure 5. Expression levels of *BTA1* and *CrDES* were recovered in the**

593 ***crbzip1-1C* and *crbzip1-2C* lines.** Supports Figures 4E and 5B.  
594 Cells were treated with 1 µg/mL tunicamycin (Tm) for the indicated periods. The RT-qPCR  
595 results were normalized by the level of *RPL17* expression and the fold changes of expression  
596 levels at 6 h were determined relative to the levels at 0 h. Error bars represent standard errors  
597 based on seven biological replicates from three individual experiments. Statistical analysis was  
598 carried out using Student's *t*-test (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; NS, no significant change) and the  
599 Wilcoxon rank sum test (Supplemental Dataset 1).

600

601 **Supplemental Figure 6. Fatty acid compositions of glycerolipids in *crbzip1* mutants**  
602 **subjected to ER stress.** Supports Figure 4A.

603 The fatty acid composition in the glycerolipids: phosphatidylinositol (PI),  
604 phosphatidylethanolamine (PE), phosphatidylglycerol (PG), sulfoquinovosyldiacylglycerol  
605 (SQDG), monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and  
606 triacylglycerol (TAG). Lipids extracted from *crbzip1* mutants and their parental strains treated  
607 with 1 µg/mL tunicamycin (Tm) for the indicated periods were analyzed using GC-FID. Error  
608 bars represent standard errors based on three biological replicates. The statistical analysis is  
609 described in Supplemental Dataset 2.

610

611 **Supplemental Figure 7. Isolation of *Chlamydomonas crdes1-1* and *crdes1-2* mutants.**

612 Supports Figure 6A.

613 (A) Schematic representation of insertion sites of the *APHVII* and *APHVIII* cassettes in the  
614 genomic sequence of *CrDES* in *crdes* mutants. The positions of UTRs, exons, and introns are  
615 indicated by dark-gray boxes, gray boxes, and lines, respectively. Arrows indicate primer  
616 locations used to detect insertions in (B).

617 (B) Genotyping of the *crdes1-1* and *crdes1-2* mutants. Genomic DNA fragments were  
618 amplified by PCR using the primer sets indicated in A.

619

620 **Supplemental Figure 8. Fatty acid compositions of glycerolipids in *crdes* mutants**  
621 **subjected to ER stress.** Supports Figure 6B.

622 Total lipids were extracted from cells treated with 1 µg/mL tunicamycin (Tm) for the indicated  
623 periods. Phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylglycerol (PG),  
624 sulfoquinovosyldiacylglycerol (SQDG), monogalactosyldiacylglycerol (MGDG),  
625 digalactosyldiacylglycerol (DGDG), triacylglycerol (TAG). Yellow boxes indicate absence or  
626 reduction of pinolenic acid in *crdes1-1* or *crdes1-2* mutants, respectively. Red boxes indicate

627 compensatory increases of alpha-linolenic acid in *crdes1* mutants. Error bars represent  
628 standard errors based on three biological replicates. Statistical analysis is described in  
629 Supplemental Dataset 3.

630

### 631 **FIGURE LEGENDS**

#### 632 **Figure 1. Identification of the transcription factor targeted by *Chlamydomonas* IRE1.**

633 (A) Molecular phylogenetic analysis of the *Chlamydomonas* bZIP proteins and the  
634 *Arabidopsis* AtbZIP60 protein reported to be important for ER stress (Nagashima et al., 2011;  
635 Deng et al., 2011). The analysis was conducted in MEGA7 (Kumar et al., 2016), using the  
636 Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). The  
637 tree is drawn to scale, with branch lengths measured in number of substitutions per site.

638 (B) Unspliced *CrbZIP1* (*CrbZIP1u*) and spliced *CrbZIP1* (*CrbZIP1s*) detected using primers  
639 flanking the spliced region of *CrbZIP1* (arrows in C). Under the ER stress conditions  
640 imposed by 1 µg/mL tunicamycin (Tm), *CrbZIP1u* is spliced to *CrbZIP1s* in CC-4533 and  
641 C-9, but not in the *crire1* mutants, *crire1-1* and *crire1-2*. *Chlamydomonas* cells were  
642 incubated in TAP medium containing 1 µg/mL Tm.

643 (C) Schematic representations of *CrbZIP1u* mRNA and *CrbZIP1s* mRNA and primer  
644 locations used to detect splicing.

645 (D) Schematic representations of primary structures of CrbZIP1u and CrbZIP1s proteins. The  
646 bZIP motif and the transmembrane domain are indicated by bZIP and TM, respectively.

647 (E) Immunoblot analysis of CrbZIP1u and CrbZIP1s using anti-CrbZIP1 antibody. Protein  
648 preparations from cells treated with 1 µg/mL tunicamycin (Tm) or 5 mM dithiothreitol (DTT)  
649 for the indicated periods were subjected to immunoblot analysis. Coomassie brilliant blue  
650 staining (CBB) was used as a loading control.

651 (F) Cellular localization of CrbZIP1u-sGFP and CrbZIP1s-sGFP transiently expressed in *N.*  
652 *benthamiana* epidermal cells. The superfolder GFP (sGFP), ER marker (AtBiP1-tagRFP),  
653 DAPI (nuclear marker), and merged images of sGFP and ER marker, DAPI, and the bright  
654 field. Note that CrbZIP1u-sGFP mainly localizes to the ER membrane and CrbZIP1s-sGFP  
655 localizes to the nucleus. Typical images of each localization pattern were chosen from each of  
656 the 6 images analyzed. Bars = 50 µm.

657

#### 658 **Figure 2. Isolation and phenotypic analysis of *Chlamydomonas crbz1p1* mutants.**

659 (A) Schematic representation of the *APHVIII* cassettes inserted into the first exon of *CrbZIP1*  
660 in the *crbz1p1* mutants. The positions of UTRs, exons, and introns are indicated by dark-gray

661 boxes, gray boxes, and lines, respectively.

662 (B) Photographs of cultures grown in control TAP medium or TAP medium containing 1  
663  $\mu\text{g}/\text{mL}$  Tm for the indicated periods.

664 (C) Cell numbers and viability of cells grown in the presence or absence of Tm. Propidium  
665 iodide (PI) was used to stain dead and damaged cells, and cells not stained with PI were  
666 considered healthy. Averages from four biological replicates from two individual experiments  
667 and their standard errors are shown.

668 (D) Confocal microscopy images of Nile red-stained cells grown in medium with 1  $\mu\text{g}/\text{mL}$   
669 Tm for 0, 6, 12, or 24 h. Images of chlorophyll autofluorescence were merged with those of  
670 Nile red fluorescence, which indicated lipid droplets.

671

672 **Figure 3. Expression levels of ER stress marker genes in *crbzip1* mutants and their**  
673 **parental strains.**

674 Cells were treated with 1  $\mu\text{g}/\text{mL}$  tunicamycin (Tm) for the indicated periods. The RT-qPCR  
675 results were normalized by the level of *RPL17* expression and the fold changes of expression  
676 levels at 2 h and 6 h were determined relative to the levels at 0 h. Error bars represent  
677 standard errors based on four biological replicates from two individual experiments.  
678 Statistical analysis was carried out using a Student's *t*-test (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ) and the  
679 Wilcoxon rank sum test (Supplemental Dataset 1).

680

681 **Figure 4. ER-stress-induced lipid remodeling differs between the *crbzip1* mutant and**  
682 **their parental lines.**

683 (A) Levels of fatty acids (FAs) in glycerolipids and the fatty acid composition of total fatty  
684 acids and of diacylglyceryltrimethylhomo-Ser (DGTS). Lipids extracted from *crbzip1*  
685 mutants and their parental strains treated with 1  $\mu\text{g}/\text{mL}$  tunicamycin (Tm) for the indicated  
686 periods were analyzed using GC-FID. Error bars represent standard errors based on three  
687 biological replicates. Red box indicates TAG accumulation in *crbzip1* cells. Green box  
688 represents the induction of DGTS only in the parental strains. Blue boxes indicate the  
689 decreases of pinolenic acid in total FAs in *crbzip1* cells. Yellow boxes indicate the dramatic  
690 reductions of pinolenic acid in DGTS. phosphatidylinositol (PI), phosphatidylethanolamine  
691 (PE), phosphatidylglycerol (PG), sulfoquinovosyldiacylglycerol (SQDG),  
692 monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and  
693 triacylglycerol (TAG). Statistical analysis was carried out using a Student's *t*-test (\*,  $p < 0.05$ ;  
694 \*\*,  $p < 0.01$ , Supplemental Dataset 2), and the significant differences between the mutant and

695 the parental strain at the same time point are marked.  
696 (B) Major TAG biosynthesis pathway mediated by DGTT1 in *Chlamydomonas*. DGTT1 is a  
697 critical enzyme for TAG synthesis in *Chlamydomonas*.  
698 (C) Expression levels of *DGTT1* under ER stress. Cells were treated with 1  $\mu\text{g}/\text{mL}$  Tm for the  
699 indicated periods. The RT-qPCR results were normalized by the level of *RPL17* expression  
700 and the fold changes of expression levels were determined relative to the levels at 0 h. Error  
701 bars represent standard errors based on four biological replicates at 0, 2, and 6 h. Statistical  
702 analysis was carried out using a Student's *t*-test (\*\*,  $p < 0.01$ ) and the Wilcoxon rank sum test  
703 (Supplemental Dataset 1).  
704 (D) DGTS biosynthesis pathway mediated by BTA1 in *Chlamydomonas*. BTA1 is a critical  
705 enzyme for DGTS biosynthesis in *Chlamydomonas*.  
706 (E) Expression level of *BTA1* in *Chlamydomonas* cells subjected to ER stress. Experimental  
707 conditions and data processing were the same as in (C).  
708 (F) Yeast one-hybrid assay showing that CrbZIP1s interacts directly with the promoter region  
709 of *BTA1*. Yeast cells were spotted on medium containing SD-Leu-Ura-His with or without  
710 3-AT with a series of 1:10 dilutions.

711

712 **Figure 5. CrbZIP1s promotes the biosynthesis of pinolenic acid (18:3 $\Delta$ 5,9,12) under ER**  
713 **stress.**

714 (A) C18 FA biosynthesis pathway in *Chlamydomonas*.  
715 (B) Relative transcript abundance of desaturase genes after tunicamycin (Tm) treatment.  
716 Cells were treated with 1  $\mu\text{g}/\text{mL}$  Tm for the indicated periods. The RT-qPCR results were  
717 normalized by the level of *RPL17* expression and the fold changes of expression levels were  
718 determined relative to the levels at 0 h. Error bars represent standard errors based on four  
719 biological replicates for 0, 2, and 6 h. Statistical analysis was carried out using a Student's  
720 *t*-test (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ) and the Wilcoxon rank sum test (Supplemental Dataset 1).  
721 (C) Yeast one-hybrid assay showing that CrbZIP1s interacts directly with the promoter region  
722 of *CrDES*. Yeast cells were spotted on medium containing SD-Leu-Ura-His with or without  
723 3-AT with a series of 1:10 dilutions.

724

725 **Figure 6. *crdes* knockdown mutants are hypersensitive to the ER stress imposed by**  
726 **tunicamycin treatment.**

727 (A) Schematic representation of insertion sites of the *APHVII* and *APHVIII* cassettes in the  
728 genomic sequence of *CrDES* in *crdes* mutants. The positions of UTRs, exons, and introns are

729 indicated by dark-gray boxes, gray boxes, and lines, respectively.  
730 **(B)** Levels of fatty acids (FAs) in glycerolipids and fatty acid composition of total fatty acids,  
731 phosphatidylethanolamine (PE), and diacylglyceryltrimethylhomo-Ser (DGTS). Total lipids  
732 were extracted from cells treated with 1  $\mu\text{g}/\text{mL}$  tunicamycin (Tm) for the indicated periods.  
733 Blue arrows and green arrows indicate absence or reduction of pinolenic acid and coniferonic  
734 acid in *crdes1-1* and *crdes1-2* mutants, respectively. Yellow boxes indicate absence or  
735 reduction of pinolenic acid in *crdes1-1* or *crdes1-2* mutants, respectively. Red boxes indicate  
736 compensatory increases of alpha-linolenic acid in *crdes1* mutants. Error bars represent  
737 standard errors based on three biological replicates. Statistical analysis was performed as  
738 described in Supplemental Dataset 3.  
739 **(C)** Growth of the indicated *Chlamydomonas* cell lines spotted on TAP medium  
740 supplemented with or without 0.2  $\mu\text{g}/\text{mL}$  Tm with a series of 1:10 dilutions.

741

742 **Figure 7. Proposed model for a mechanism of ER stress responses in *crbzip1* mutants**  
743 **and their parental lines in *Chlamydomonas*.**

744 Left: In the parental lines, activated CrIRE1 splices *CrbZIP1u* mRNA to produce *CrbZIP1s*,  
745 which is translated to CrbZIP1s. CrbZIP1s enters the nucleus, where it induces the expression  
746 of UPR genes and ER membrane lipid biosynthesis genes, such as *BTA1* and *CrDES*. The  
747 dimerization and phosphorylation of CrIRE1 are drawn based on reports in other organisms  
748 but have not been revealed in *Chlamydomonas*.

749 Right: The *crbzip1* mutation upregulates TAG biosynthesis from the fatty acids hydrolyzed  
750 from a major ER membrane lipid, DGTS, resulting in the formation of lipid droplets, which  
751 store TAGs.

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