1 **Short Title:** Interplay between autophagy and lipid metabolism

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33 **Title:**

34 **Autophagy is required for lipid homeostasis during dark-induced senescence in**
 35 **Arabidopsis**

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49 **One-sentence summary:** Autophagy is required for the mobilization of membrane lipid
 50 components and lipid droplet dynamics during extended darkness in *Arabidopsis*.

51

52 **Author contributions:**

53 J.A.S.B., W.L.A., and T.A-W.. designed the research; J.A.S.B. performed most of the
 54 research; T.A-W. and W.L.A. supervised the project; Y.B. contributed with new
 55 reagents/analytic tools for lipid analysis; S.M contributed with transcript and western
 56 blot analysis; T.L-C performed lipid profile; L.R. performed the free fatty acid analysis;
 57 J.A.S.B., W.L.A., and T.A-W. analyzed the data and wrote the article with contributions
 58 of all the authors.

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68 **Abstract**

69 Autophagy is an evolutionarily conserved mechanism that mediates the degradation of
70 cytoplasmic components in eukaryotic cells. In plants, autophagy has been extensively
71 associated with the recycling of proteins during carbon starvation conditions. Even
72 though lipids constitute a significant energy reserve, our understanding of the function
73 of autophagy in the management of cell lipid reserves and components remains
74 fragmented. To further investigate the significance of autophagy in lipid metabolism,
75 we performed an extensive lipidomic characterization of *Arabidopsis* (*Arabidopsis*
76 *thaliana*) autophagy mutants (*atg*) submitted to dark-induced senescence conditions.
77 Our results revealed an altered lipid profile in *atg* mutants, suggesting that autophagy
78 affects the homeostasis of multiple lipid components under dark-induced senescence.
79 The acute degradation of chloroplast lipids coupled with the differential accumulation
80 of triacylglycerols (TAGs) and plastoglobuli indicates an alternative metabolic
81 reprogramming towards lipid storage in *atg* mutants. The imbalance of lipid metabolism
82 compromises the production of cytosolic lipid droplets and the regulation of
83 peroxisomal lipid oxidation pathways in *atg* mutants.

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100 Introduction

101 The precise control of energy homeostasis is crucial for plant survival,
102 particularly under biotic and abiotic stress conditions. Under low energy availability,
103 plants require a strategy other than carbohydrate oxidation to fully sustain energy
104 production. Therefore, proteins, lipids, and chlorophylls can be catabolized instead of
105 sugars to provide alternative substrates for the tricarboxylic acid (TCA) cycle. This
106 assures the continued operation of the mitochondrial electron transport chain (Ishizaki et
107 al., 2005; Kunz et al., 2009; Araujo et al., 2011; Hortensteiner & Krautler, 2011).

108 Among the alternative substrates of respiration in plants, lipids comprise a
109 significant carbon reserve that represents an important alternative source of energy.
110 Although in comparison to carbohydrates, the usage of lipids is less efficient, as
111 assessed by the respiratory quotient, their intracellular storage and utilization are critical
112 for the maintenance of cellular energy homeostasis (Araujo et al., 2011; Yang et al.,
113 2018). Plants store lipids as the neutral lipid species TAG (Yang et al., 2018). In leaf
114 mesophyll cells, TAGs are deposited in specialized storage compartments, namely lipid
115 droplets (LDs) in the cytosol or plastoglobuli (PG) inside chloroplasts (Van wick et al.,
116 2017). During nutrient deprivation, cellular lipid reserves are hydrolyzed into fatty acids
117 (FAs), which are further catabolized during β -oxidation. Accordingly, mutants with
118 dysfunctional β -oxidation, triggered by the disruption of the PEROXISOMAL ABC
119 TRANSPORTER 1 (PXA1), exhibit hypersensitivity to extended darkness (Kunz et al.,
120 2009). Moreover, several transcripts of the β -oxidation pathway are up-regulated during
121 developmental leaf senescence (Troncoso-Ponce et al., 2013).

122 Autophagy, one of the primary cellular catabolic process, is involved in the
123 degradation and recycling of cytoplasmic constituents into primary metabolites (Avin-
124 Wittenberg et al., 2018). The autophagy mechanism is governed by autophagy-related
125 (ATG) genes that are highly conserved among eukaryotes (Marshall and Vierstra, 2018).
126 Plants with genetic disruption of ATG genes (hereafter *atg* mutants) have been well
127 characterized for sensitivity to several stresses, particularly nutrient starvation (Avin-
128 Wittenberg, 2018). The importance of autophagy in plants has been mostly associated
129 with the recycling of proteins to amino acids under starvation conditions (Izumi et al.,
130 2013; Li et al., 2014; Barros et al., 2017; Hirota et al., 2018; McLoughin et al., 2018,
131 2020). By contrast, in mammals and yeast, autophagy has been largely associated not
132 only with proteins but also with lipid metabolism (Elander et al., 2018; Barros et al.,
133 2020). Accordingly, it was shown that autophagy supplies FAs for LD biogenesis as

134 well as mediates the selective degradation of LDs in both mammals and yeast (Singh et
135 al., 2009; Rambolt et al., 2015; Ngyugen et al., 2017).

136 A growing body of evidence suggests a dual role of autophagy in the lipid
137 metabolism of alga and plants. Thus, in *Chlamydomonas* (*Chlamydomonas reinhardtii*),
138 it has been postulated that autophagy participates in both TAG biosynthesis and LD
139 formation (Couso et al., 2017; Pugkaew et al., 2017). In addition, microscopic evidence
140 pointed to the autophagy-mediated engulfment of LDs in the algae *Auxenochlorella*
141 *protothecoides* (Zhao et al., 2014) and *Micrasterias denticulata* (Schwarz et al., 2017).
142 Interestingly, recent data also suggested that autophagy may mediate LD formation
143 during an early stage of starvation while likely operating in LD degradation at the later
144 stages of nitrogen starvation in *Chlamydomonas* (Tran et al., 2019).

145 In vascular plants, it was demonstrated that autophagy contributes to TAG
146 synthesis via the turnover of organellar membranes in *Arabidopsis* under normal growth
147 conditions (Fan et al., 2019). On the other hand, it was suggested the autophagy
148 operates in the degradation of LDs by a selective microautophagy mechanism under
149 extended darkness (Fan et al., 2019). Notably, this study was based on the analysis of
150 *Arabidopsis* mutants overexpressing the LD marker OLE1-GFP and knockout for
151 SUGAR DEPENDENT-1 (SDP1) lipase, which are genotypes with altered LD and
152 TAG metabolism (Fan et al., 2013, 2017; Barros et al., 2020). In maize (*Zea mays*),
153 *atg12-1* mutant presented a higher LD number, which could reflect active LD assembly
154 or disruption of LD catabolism (McLoughin et al., 2020). It remains contentious,
155 however, whether autophagy mediates the biogenesis or degradation of LDs in
156 vegetative tissues (Elander et al., 2018; Barros et al., 2020; Masclaux-Daubresse et al.,
157 2020). Although recent studies have clearly revealed an interplay between autophagy
158 and lipid metabolism (McLoughin et al., 2018, 2020; Have et al., 2019; Fan et al., 2019),
159 relatively little is known about the role of autophagy in the mobilization of lipid
160 components under energy limitation conditions.

161 Here, we investigated the significance of autophagy in lipid metabolism during
162 dark-induced senescence in *Arabidopsis*. By performing an extensive lipidomic analysis
163 of three independent *atg* mutants, we observed an overall impact of autophagy
164 deficiency on all lipid classes analyzed and the free fatty acid (FFA) profile. The
165 differential pattern of phospholipid levels and TAG accumulation was further
166 coordinated with changes in LD number and PG size. In addition, analysis of β -
167 oxidation genes revealed an induction of lipid oxidation pathways in *atg* mutants.

168 Collectively, our results demonstrate that autophagy is an essential component in the
169 management of lipid reserves, affecting membrane lipid mobilization, the balance of LD
170 and PG formation, and the regulation of β -oxidation pathways under dark-induced
171 senescence.

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174 Results

175 Differential lipid response of *atg* mutants under dark-induced senescence

176 To examine the involvement of autophagy in lipid remodeling during dark-
177 induced senescence, we analyzed three previously described Arabidopsis autophagy
178 mutants: *atg5-1* and *atg7-2* with full inhibition of autophagy (Chung et al., 2010); and
179 *atg9-4* mutant that displayed a milder reduction of the autophagic process (Shin et al.,
180 2014). The experimental setup consisted of the submission of 4-week-old plants to
181 extended darkness conditions, a system widely used to induce energetic stress in plants
182 (Ishizaki et al., 2005; Araujo et al., 2010; Barros et al., 2017).

183 To further investigate the impact of autophagy disruption on lipid homeostasis
184 during dark-induced senescence, we performed Liquid Chromatography–Mass
185 Spectrometry lipidomic analysis of *atg5-1*, *atg7-2*, *atg9-4*, and their corresponding
186 wild-type (WT) plants. We were able to identify 119 lipid species of the following
187 classes: Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), Diacylglycerol
188 (DAG), Triacylglycerol (TAG), Monogalactosyldiacylglycerol (MGDG), and
189 Digalactosyldiacylglycerol (DGDG). We first performed a principal component analysis
190 (PCA) based on the lipid profiles of samples from control and 6 days (d) of darkness
191 (Fig. 1). Most of the variance of the dataset was covered by the first two principal
192 components, explaining 69.1% of the overall data variance (57.8% and 11.3% for PC1
193 and PC2, respectively). This fingerprint analysis allowed us to obtain a general picture,
194 discerning the main differences between the darkness treatment and the *atg* mutant
195 lines. Briefly, it revealed that under control conditions (before darkness treatment), all
196 genotypes clustered together; however, a clear separation according to the treatment and
197 genotype was observed following darkness, wherein *atg7-2* and *atg5-1* were
198 disconnected of the WT samples (Fig. 1). Interestingly, *atg9-4* presented an
199 intermediary distribution, sharing certain metabolic similarities with WT and the other
200 *atg* genotypes. The analysis of all data points also highlighted the delayed response of
201 *atg9-4*, which after 6d and 9d of darkness, was more closely related to *atg5-1* and *atg7-2*
202 after 3d of darkness conditions (Supplemental Figure S1).

203 Our results are in close agreement with the milder impact of the *ATG9*
204 disruption on metabolic features previously reported under dark-induced senescence
205 (Barros et al., 2017). This response was related to residual autophagy activity observed
206 in *atg9* lines (Yoshimoto et al., 2004; Shin et al., 2014), which explains the intermediary
207 lipid response of *atg9-4* genotype. The information of the individual variables that

Figure 1 - Differential lipid response of *atg* mutants under dark-induced senescence.

Principal component analysis (PCA) was performed using the lipid data obtained in samples from 4-week-old *Arabidopsis* plants immediately before lights were turned off (0 d) and after further treatment for 6d in darkness. Three loss-of-function mutants of the autophagy pathway, namely *atg5-1*, *atg7-2*, *atg9-4* and their respective wild type (WT) were analyzed.

208 contributed most to PC1 and PC2 (i.e., the lipids with the main impact on the variance
209 of the dataset) is additionally available in Supplemental Table S1. Several lipids
210 accounted for the main changes observed in the PCA. Briefly, PC1 was mainly
211 comprised of MGDG 34:4, MGDG 34:3, PC 38:4(1) and MGDG 34:1 underlining the
212 separation of darkened and control samples. On the other hand, TAG 54:9, MGDG 32:3
213 (1), and TAG 52:6 were the main lipids responsible for changes in PC2 (Supplemental
214 Table 1), contributing to the differences between the genotypes under darkness (Fig. 1).

215

216 **The lack of autophagy affects the dynamics of phospholipids during dark-induced** 217 **senescence**

218 In order to obtain a more detailed characterization of the lipid composition of
219 *atg* mutants following extended darkness, we analyzed each lipid class separately. We

Figure 2 - *atg* mutants present altered phospholipid levels under control and extended darkness conditions

Hierarchical clustering of (A) Phosphatidylcholine (PC) and (B) Phosphatidylethanolamine (PE). Values plotted are log₂ fold change. Distances were measured by Pearson correlation using MeV 4.9.0 software. Data were normalized to the mean response calculated for the 0-d dark treated leaves of the wild type (WT). Values presented are means $\pm$ SE of five-six biological replicates per genotype; an asterisk (*) designates values that were determined by Student's t-test to be significantly different ($P < 0.05$) from WT at each time point analyzed.

220 were able to identify the two dominant structural phospholipid species, namely PC and
 221 PE. These lipids are components of the plasma membrane, in which PC is associated
 222 with the outer leaflet, and PE is concentrated in the inner leaflet. PC and PE are also
 223 precursors of lipid mediators, such as phosphatidic acid (PA) and diacylglycerol (DAG)
 224 (Wang et al., 2006; Testerink and Munnik, 2011). Additionally, PE plays a crucial role
 225 in autophagosome formation (Soto-Burgo et al., 2018; Barros et al., 2020).
 226 Interestingly, a slight accumulation of both PC and PE was observed in *atg5-1* and *atg7-2*
 227 mutants before darkness treatment (control conditions, Fig. 2 – 0d). The difference of
 228 PC levels under control conditions was marked by the accumulation of 36C PC in *atg5-1*
 229 and *atg7-2* mutants, whereas no significant changes were observed in *atg9-4* at this
 230 time point (Fig. 2A – 0d). Extended darkness triggered a mixed response of different PC
 231 species. Thus, the levels of 32C and 34C mostly increased in WT plants under darkness.
 232 However, *atg5-1* and *atg7-2* presented lower accumulation of 34C PC species that
 233 contain a combination of 18C and 16C polyunsaturated acyl species, such as 34:5-
 234 (18:3-16:2-) and 34:6- (18:3-16:3) during dark treatment. The PC containing FAs with
 235 longer chains, 36C and 38C, were generally reduced in response to extended darkness.

236 However, *atg5-1* and *atg7-2* presented a milder reduction of 36C PC species after 3d
237 and 6d of darkness, whereas the 38C species (38:3(2), 38:4, 38:5, 38:6) were more
238 reduced in these genotypes after 6d and 9d of darkness (Fig 2A).

239 Following dark treatment, the majority of PE species decreased in WT plants;
240 however, this reduction was milder in *atg* mutant lines after 6d of darkness (Fig. 2B).
241 On the other hand, after 9d of darkness, species such as PE 36:2, 34:2, and 36:3(2) were
242 highly reduced in *atg5-1* and *atg7-2* compared to WT. Surprisingly, the PE species 36:5
243 and 38:3 presented a contrasting pattern, accumulating after 6d and 9d of darkness in
244 *atg* mutants (Fig. 2B).

245

246 **Impairment of autophagy accelerates the loss of chloroplast lipids**

247 Since chloroplast lipids are subjected to constant turnover during senescence, we
248 next evaluated the predominant lipid constituents of chloroplast membranes, MGDG
249 and DGDG. An apparent reduction in more than 90% of the detected MGDGs and
250 DGDGs was observed in *atg* mutants exposed to dark treatment (Fig. 3). This response
251 was intensified throughout the dark treatment, and thus the levels of some species were
252 below the detection threshold after 9d. Although *atg9-4* mutants followed the same
253 pattern, the levels of MGDG and DGDG were less reduced in comparison to *atg5-1*
254 and *atg7-2* mutants (Fig. 3). The overall reduction of chloroplast lipids reported here is
255 consistent with the more severe phenotype described previously for *atg5-1* and *atg7-2*
256 mutants lines under extended darkness (Barros et al., 2017). Interestingly, slightly lower
257 levels of unsaturated MGDG and DGDG were also observed in *atg* mutants under
258 control conditions, highlighting the potential role of autophagy in basal chloroplast
259 maintenance, as recently verified in different studies using both *Arabidopsis atg5* and
260 maize *atg12* mutants (McLoughlin et al., 2018, 2020; Have et al., 2019).

261

262 **Disruption of autophagy compromises TAG and LD response under dark-induced** 263 **senescence**

264 The analysis of neutral lipids revealed a general increase in TAG levels during
265 extended darkness. TAGs are composed of three FAs esterified to a glycerol backbone
266 (Yang et al., 2018). The dominating accumulated TAG species during dark-induced
267 senescence harbor three FA molecule with 18C with two or three double bonds (54:7 to
268 54:9 in Fig. 4A), or two FA molecules with 18C with two to three double bonds and
269 16:3 or 16:0 as a third FA (52:5 to 52:9 in Fig. 4A). Unsaturated C18 FAs (oleic acid,

Figure 3 - Chloroplast lipids are substantially reduced in *atg* mutants during dark-induced senescence
Monogalactosyldiacylglycerol (MGDG) and Digalactosyldiacylglycerol (DGDG) values plotted are log₂ fold change. Data were normalized to the mean response calculated for the 0-d dark treated leaves of the wild type (WT). Values presented are means $\pm$ SE of five-six biological replicates per genotype; an asterisk (*) designate values that were determined by the Student's t-test to be significantly different ($P < 0.05$) from WT at each time point analyzed.

18:1; linoleic acid, 18:2, and α -linolenic acid, 18:3), palmitic acid (16:0) and unsaturated C16 FAs (16:1 and 16:3) are highly abundant in chloroplasts of Arabidopsis (Hölzl and Dörmann, 2019). Our data indicate that the accumulated TAG species are most likely derived from plastidial lipids. Interestingly, the increase in chloroplast-derived TAG species was less pronounced in both *atg5-1* and *atg7-2* mutants after 3d and 6d of darkness. Additionally, an increase of other TAG species was observed from 6d of dark treatment onwards in *atg5-1* and *atg7-2* mutants, resulting in general TAG accumulation during the late period of darkness (Fig. 4A).

DAG is a central metabolite in plant lipid metabolism, serving as an intermediate of both TAG and galactolipids (Muthan et al., 2013). Similar to the observed for TAGs, the four DAG molecules identified (34:3, 34:6, 36:5, 36:6) were composed of unsaturated FAs, containing at least one unit of 16:2, 16:3 or 18:3 plastid-derived FA (Fig. 4B). Although no significant differences were observed during the early stages of dark treatment, DAGs started to accumulate 6d after the onset of dark treatment in *atg5-1* and *atg7-2* mutants. At the same time, WT and *atg9-4* plants showed a trend of reduction (Fig. 4B). The accumulation of unsaturated DAG species is likely a result of the degradation of chloroplast membranes in *atg* mutants serving as a

Figure 4 - Distinct accumulation of neutral lipids in *atg* mutants during dark-induced senescence
 (A) Triacylglycerol (TAG) and (B) Diacylglycerol (DAG). Values plotted are $\log_2$ fold change. Data were normalized to the mean response calculated for the 0-d dark treated leaves of the wild type (WT). Values presented are means $\pm$ SE of five-six biological replicates per genotype; an asterisk (*) designates values that were determined by Student's t-test to be significantly different ($P < 0.05$) from WT each time point analyzed.

287 precursor for the general TAG accumulation in the late stages of dark-induced
 288 senescence.

Figure 5 - Autophagy deficiency affects Lipid Droplet (LD) dynamics during dark-induced senescence
 (A) Representative confocal images of LDs (BODIPY 493/503 fluorescence, false color (green) in wild type (WT), *atg5-1*, and *atg7-2* leaves. (B) Merged image of BODIPY and chloroplast autofluorescence (false color red). Images were collected at the same magnification and are projections of Z-stacks of 20 optical sections Bar = 10 μ m (C) Number of total LDs per image area. The number of LDs was quantified by ImageJ as BODIPY-stained lipid area in 2D projections of Z-stacks of 3 images of four biological replicates per genotype and time point.

289 TAG is the central lipid reserve of the plant cell, stored in LDs (Pyc et al.,
 290 2017). To further investigate how the altered levels of TAGs in *atg7-2* and *atg5-1*
 291 mutants affect LD metabolism during dark-induced senescence, we visualized the
 292 abundance of LDs in leaf tissues by staining with the neutral-lipid-selective fluorescent
 293 dye, BODIPY493/503. An increased number of LDs was observed after 3d of darkness
 294 in WT plants (Fig. 5). However, no accumulation of LDs was observed in *atg5-1* and
 295 *atg7-2* mutants (Fig. 5). Notably, the multiple z-stacked confocal images revealed that
 296 LDs are mostly accumulated in the cytosol and not inside chloroplasts (Fig. 5A). The
 297 dynamics of LD accumulation corroborates the high levels of the plastid-derived TAG
 298 species, which were subsequently reduced after 6d of darkness in WT. Furthermore,
 299 *atg5-1* and *atg7-2* mutants displayed reduced levels of plastid-derived TAG species that
 300 can also be associated with the impairment of LD accumulation (Fig. 4A).

301

302 The disruption of autophagy triggers activation of PG metabolism

303 PG are specialized globular LDs composed by a membrane lipid monolayer
 304 surrounding a core of neutral lipids present in the chloroplast membranes (Van Wijck an
 305 Kessler, 2017). The production of PG is related to the accumulation of products from

306 chlorophyll and chloroplast lipid catabolism such as fatty acid phytol esters (FAPEs),
 307 TAGs and FAs (Gaude et al., 2007; Lippold et al., 2012). Given that *atg* mutants
 308 presented a substantial reduction of chloroplast lipids under extended darkness, which
 309 was not translated to an increase in TAGs or LD accumulation, we next investigated
 310 PG-related pathways by quantitative reverse transcription (RT)-PCR. The genes phytol
 311 ester synthase 1 (*PES-1*) and *PES-2*, belonging to the esterase/lipase/thioesterase family,
 312 are required for FAPE production in Arabidopsis PG during senescence (Lippold et al.,
 313 2012). Accordingly, we observed an increase of *PES-1* and *PES-2* transcript levels
 314 following dark-induced senescence (Fig. 6A-B). However, higher induction of *PES-1* in
 315 *atg5-1* and *atg7-2*, as well as of *PES-2* in *atg7-2* mutant, was observed after 3d and 6d

Figure 6 - PG related genes are up-regulated in *atg* mutants during dark-induced senescence

Transcript abundance is shown for genes associated with plastoglobuli (PG), including *PES-1* (A), *PES-2* (B), *ABC1K7* (C), *PPH* (D). The y-axis values represent the gene expression level relative to the wild type (WT). Data were normalized with respect to the mean response calculated for the 0-d dark-treated leaves of the WT. Values presented are means $\pm$ SE of three independent biological replicates. An asterisk (*) indicates values that were determined by Student's t-test to be significantly different ($P < 0.05$) from the WT at each time point analyzed. A number sign (#) indicates values determined by Student's t-test to be significantly different ($P < 0.05$) from WT at 0d.

316 of darkness (Fig. 6A-B). The production of FAPE relies on both FFA released from
 317 chloroplast membranes and phytol from chlorophyll (Lippold et al., 2012). We,
 318 therefore, analyzed the chlorophyll degradation enzyme, pheophytin pheophorbide
 319 hydrolase (*PPH*). As observed for *PES1* and *PES2*, the *PPH* transcript accumulated at
 320 higher levels in *atg* mutants under dark-induced senescence in comparison to WT levels
 321 (Fig. 6D).

322 PG serve not only as a lipid deposit but also contain an actively associated
 323 proteome (Lundquist et al., 2012). The presence of several Absence of Bc1 Complex

Kinase (ABC1K) proteins was described in PG, suggesting a central role of these proteins in the regulation of PG metabolism (Ytterberg et al., 2006; Brehelin et al., 2007). The protein ABC1K7 has been characterized as a PG core component with marked accumulation during late senescence events (Lundquist et al., 2012; Bhuiyan et al., 2016). Despite the unchanged transcript levels of *ABC1K7* in WT, this gene was induced in *atg5-1* and *atg7-2* mutants following extended darkness (Fig. 6C). Although our results demonstrate a boost of PG pathways in the absence of autophagy under dark-induced senescence, *atg* mutants presented a slight but significant up-regulation for all PG-related genes even under control conditions (Fig. 6), highlighting intrinsic crosstalk between these processes.

Transmission electron microscopy analysis revealed that the number and size of PG as well as the chloroplast structure are similar in leaves of WT and *atg5-1* plants under control conditions (Figure 7A). After dark treatment, different chloroplast responses were observed in WT and *atg5-1* plants (Figure 7A). While WT presented a typical thylakoid stacking response of darkened chloroplast (Wood et al., 2019), *atg5-1* displayed smaller chloroplasts with disrupted structural remodeling, containing structures resembling senescence-associated vacuoles (Otegui et al., 2005). Moreover, a marked presence of PG was observed in *atg5-1* chloroplasts (Figure 7A). This response was ascribed to an increase in the size of PG, which reached up to $0.02 \mu\text{m}^2$ of area (Figure 7B-C). Otherwise, WT plants also presented increases in PG size, however, to a far less extent compared to *atg5-1* mutant (Figure 7B-C).

Proteomic studies of PG have shown that members of the Fibrillin (FBN) family are the most abundant proteins in the PG proteome (Lundquist et al., 2012). Therefore, we next checked the content of the PG core protein FBN1a (Fibrillin 1a), previously characterized as a PG structural component (Austin 2006, Simkin et al., 2007, Singh et al., 2011). Immunoblotting analysis showed a strong reduction of FBN1a abundance in *atg5-1* mutant compared to WT, which display a slight increase of FBN1a abundance after 6d of dark treatment (Figure 7D and Supplemental Figure S2). Interestingly, the overexpression of FBN1a homologs resulted in increased numbers and clustering of PGs in tobacco leaves and tomato fruit (Rey et al., 2000; Simkin et al., 2007). Additionally, FBN overexpression was also associated with the delay of thylakoid disintegration, suggesting its role on protection/maintenance of chloroplast membranes (Simkin et al., 2007; Rottet al., 2015). The reduction of FBN1a protein abundance is most likely related to the occurrence of supersized PG coupled with the overall

358 degradation of chloroplast membranes in *atg5-1* mutant plants under dark-induced
 359 senescence. Collectively, our findings highlight PG as a potential lipid storage site in
 360 *atg* mutants submitted to extended darkness conditions. It will therefore be interesting to
 361 determine hereafter whether and which extent FBNs determine PG structural modeling
 362 during low energy conditions.
 363
 364 ***atg* Mutants induce lipid oxidation pathways in response to dark-induced**
 365 **senescence**

FA oxidation may be of particular significance when the carbon and energy status is rather low. During carbon starvation, the importance of lipid metabolism was demonstrated by the characterization of β -oxidation loss-of-function mutants (Kunz et al., 2009). In addition, autophagy association with peroxisomal protein abundance was recently demonstrated (McLoughlin et al., 2018, 2020; Have et al., 2019). The general reduction of chloroplast lipids, together with reduced LD numbers in *atg5-1* and *atg7-2* mutants subjected to extended darkness, raised the question of whether these lipids were being quickly oxidized to be used as energetic substrates in *atg* mutants. We, therefore, evaluated the expression of genes related to LD catabolism and peroxisomal pathways of FA oxidation. We observed that the transcript levels of the peroxisomal enzymes

Figure 7: Autophagy disruption triggers altered PG response

(A) Transmission electron micrographs of leaves from 4-week-old Arabidopsis plants immediately before lights were turned off (0 d) and after further treatment for 7 days of darkness. Scale bar = 1 μ m; Quantification of the number (B) and size (C) of plastoglobuli per chloroplasts ($\pm$ SE, n= 6-7 chloroplasts of three independent cells); Analysis shown in A-C were performed in *atg5-1* and its respective wild type (WT). (D) Immunoblot analysis of the PG protein, FBN1a, was performed in leaves from 4-week-old Arabidopsis *atg5-1*, *atg7-2* and WT plants immediately before (0 d) and after further treatment for 3d and 6d in darkness. FBN1a protein display a predicted molecular mass to ~30-35 kD. The detection of a second lower band in the region of 30kD possibly corresponds to FBN1b protein (with high sequence similarity with FBN1a) as previously observed in Giacomelli et al., 2006; Matinis et al., 2013; 2014.

Acyl-CoA oxidase (*ACX-4*), Multifunctional protein 2 (*MPF-2*), Ketoacyl-CoA thiolase (*KAT-2*), Citrate synthase 1 (*CSY-1*) and peroxisomal Malate Dehydrogenase (*pMDH1*) genes were generally higher in *atg5-1* and *atg7-2* under extended darkness (Fig. 8 A-E). More specifically, *ACX-4* and *CSY-1* were slightly induced in WT throughout the treatment. However, higher transcript levels were observed in *atg5-1* and *atg7-2* plants

after 6d of darkness (Fig. 8A-B). Despite no major changes in *MPF-2* and *KAT-2* transcript abundance in WT plants, trends of increase were observed in *atg5-1* and *atg7-2* after 9d of darkness (Fig. 8C-D). We further confirmed that *pMDH-1* was downregulated following dark-induced senescence. *pMDH-1* operates in the reduction of oxaloacetate to malate and enables the re-oxidation of NADH for continued β -oxidation (Pracharoenwattana et al., 2007). Given that *pMDH-1* is not participating directly in β -oxidation, it is possible that an alternative pathway of NADH oxidation becomes operational under carbon starvation.

Figure 8 - Changes in transcript levels in WT and *atg* mutants during dark-induced senescence. Transcript abundance is shown for genes associated with lipid catabolic pathways, including *ACX-4* (A), *CSY-1*(B) *KAT-2* (C), *MPF-2* (D), *pMDH-1* (E), *SDP-1* (F). The y-axis values represent the expression level relative to the wild type (WT). Data were normalized with respect to the mean response calculated for the 0-d dark-treated leaves of the WT. Values presented are means $\pm$ SE of three independent biological replicates. An asterisk (*) indicates values that were determined by Student's t-test to be significantly different ($P < 0.05$) from the WT at each time point analyzed. A number sign (#) indicates values determined by Student's t-test to be significantly different ($P < 0.05$) from WT at 0d.

To investigate whether the reduced number of LDs is a result of the induction of catabolic pathways or impairment in biosynthesis, we next evaluated the expression of *SDP-1* gene. Despite being characterized as the primary lipase mediating LD catabolism in seeds, compelling evidence has suggested that *SDP-1* also operates in senescent and carbon-starved leaves (Fan et al., 2017, 2019). Indeed, an accumulation of *SDP-1* transcript levels was observed in WT plants during the early stages of darkness (Fig. 8F). Nonetheless, no significant difference in *SDP-1* transcript levels between WT plants and *atg5-1* and *atg7-2* was observed, suggesting that the reduced levels of LDs

are likely more related to the impairment of LD biogenesis rather than an induction of LD degradation pathways.

Altered FFA response of *atg* mutants under dark-induced senescence

Since autophagy deficiency altered cytosolic LD dynamics and β -oxidation pathways, we next evaluated if this response also affected the pool of free FA (FFA). Figure 9 shows that long-chain FFA (between 13C-21C chain length) are the more affected by both genotype and darkness, while no major changes were observed for very long-chain FFA (> 22C). Higher levels of 16:3, 18:1, 18:2, 18:3 in *atg5-1* and *atg7-2* genotypes under control conditions were observed, reinforcing the functional role of autophagy in basal lipid homeostasis. Upon darkness treatment, the levels of C18 and C20 FFAs were reduced, and unsaturated C16 accumulated in *atg5-1* and *atg7-2* mutants (Fig. 9). By contrast, WT plants presented an initial increase followed by later reduction of C16 and C18 FFAs except for 16:3 and 18:1 species FFAs, which were respectively, accumulated and reduced throughout darkness (Fig. 9). Although unsaturated C16 and C18 FAs are typical components of chloroplast membranes, only 16:3 is exclusively found in the thylakoid lipid MGDG in plant species (e.g. Arabidopsis), and it is, therefore, a chloroplast lipid marker (Ohlrogge et al., 1991; Kunz et al., 2009; Hölzl and Dörmann, 2019). Thus, the overall accumulation of 16:3 is in good agreement with the reduction of galactolipids observed under dark-induced senescence. Additionally, this result suggests that the rate of 16:3 release exceeds its channeling to LD and PG. The higher accumulation of 16:3 in *atg* mutants strengthens our claim that this disbalance is more intense when autophagy is disrupted.

Figure 9- Changes in free Fatty Acid levels in WT and *atg* mutant plants under dark-induced senescence.

Values plotted are log₂ fold change. Data were normalized to the mean response calculated for the 0-d dark treated leaves of the wild-type (WT). Values presented are means ± SE of five-six biological replicates per genotype; an asterisk (*) designate values that were determined by the Student's t-test to be significantly different ($P < 0.05$) from WT each time point analyzed.

422 Discussion

423 It is well established that plant autophagy plays a crucial role in the energetic
 424 metabolism mediating the supply of proteins and amino acids under carbon starvation
 425 conditions (Avin-Wittenberg et al., 2015; Barros et al., 2017; Hirota et al., 2018;
 426 McLoughlin et al., 2020). Furthermore, in both animals and yeast, autophagy is
 427 recognized as an important lipid catabolic pathway in starved cells (Singh et al., 2009).
 428 In plant leaves, growing evidence has highlighted the role of autophagy in managing
 429 cell lipid components and reserves (Avin-Wittenberg et al., 2015; McLoughlin et al.,
 430 2018,2020; Fan et al., 2019; Have et al., 2019). In fact, the lipid response of *atg* mutants
 431 under N starvation has been previously demonstrated (McLoughlin et al., 2018; Have et
 432 al., 2019). More recently, McLoughlin et al. (2020) reported extensive lipid alterations
 433 in maize *atg12* mutants submitted to carbon deprivation induced by short-term
 434 darkness. Here, we provide further evidence that autophagy broadly affects lipid and LD
 435 metabolism during dark-induced senescence in Arabidopsis. Notably, similarities and
 436 differences between the aforementioned studies and the findings reported here were

437 observed. The main points were summarized in Supplemental Figure S3, and they will
438 be further discussed throughout the text.

439 By performing a detailed lipidomics analysis, we revealed that autophagy widely
440 affects the lipid homeostasis of Arabidopsis during dark-induced senescence. The major
441 differences reported here are related to the *atg5-1* and *atg7-2* mutant plants,
442 characterized by a full inhibition of autophagy (Chung et al., 2010). By contrast, *atg9-4*,
443 which is characterized by a partial reduction of autophagy, presented an intermediary
444 impact on lipid metabolism that was more evident during the late stages of extended
445 darkness (Fig. 1 and Supplemental Figure S1). The fact that *atg9-4* did not cluster with
446 *atg5-1* and *atg7-2* implies that the lipid response is directly related to autophagy
447 intensity. Indeed, the characterization of Arabidopsis *ATG5* and *ATG7* knockout and
448 overexpression mutants showed a positive correlation between the level of autophagy
449 and seed FA content (Minina et al., 2018). We previously demonstrated that amino acid
450 catabolic pathways were less compromised in *atg9-4* mutants, contributing to its more
451 tolerant phenotype during dark-induced senescence (Barros et al., 2017). Additionally,
452 the limited reproductive phenotype of *atg* mutants is also dependent on autophagy
453 intensity since parameters related to seed and silique production were more
454 compromised in *atg* lines fully defective in autophagy (Barros et al., 2017). Taking into
455 account the fact that Arabidopsis is an oilseed (Li et al., 2006), the results reported here
456 and by others (Minina et al., 2018) suggest that the altered reproductive and carbon
457 starved phenotype of *atg* mutants is likely not only a consequence of impairment of
458 protein catabolic process but is also derived from an extensive reprogramming of lipid
459 metabolism.

460 The detailed analysis of the specific lipid classes revealed an altered lipid
461 phenotype in mutants lacking autophagy under non-stress conditions. These differences
462 are mostly attributed to galactolipids and the phospholipids PC and PE, whereas minor
463 differences were observed for TAGs (Figs 2, 3, and 4). The galactolipids MGDG and
464 DGDG are prevalent in the chloroplast envelope and the thylakoid membrane (Hölzl
465 and Dörmann, 2019). By contrast, PC and PE are highly abundant in the plasma
466 membrane, mitochondrial membrane, and endoplasmic reticulum (Liu et al., 2015).
467 Hence, the high levels of PC and low levels of MGDG and DGDG reveal an imbalance
468 of cytosolic and plastidial membrane lipids in *atg5-1* and *atg7-2* mutants under normal
469 growth conditions. Similar results were previously reported in Arabidopsis *atg5*
470 mutants, which displayed a general alteration of phospholipid levels under non-stressful

471 conditions (Have et al., 2019). The accumulation of lipid-breakdown products in maize
472 *atg12* mutants also suggested an induced turnover of membrane components under
473 normal growth conditions (McLoughlin et al., 2018, 2020). In addition, Fan et al., 2019
474 reported that the disrupted membrane lipid turnover in *atg* mutants affected TAG
475 synthesis. The TAG profile detailed here reveals that only specific TAG species are
476 reduced in *atg5-1*, and *atg7-2* mutants under normal growth conditions, which seems to
477 contribute to the low total TAG content previously reported.

478 Collectively, our results support the notion that, beyond the role of autophagy in
479 basal lipid metabolism, autophagy is clearly required for lipid remodeling under
480 starvation conditions in Arabidopsis. Upon exposure to extended darkness, PC
481 metabolism is broadly rearranged, presenting different patterns of accumulation and
482 reduction (Fig. 2). In general, the majority of 32C and 34C PC species increases,
483 whereas 36C and 38C declines upon darkness (Fig. 2). However, an altered response of
484 PC accumulation and degradation was observed in *atg* mutants. The turnover of
485 membrane lipids is a recognized aspect of plant senescence in general (Troncoso-Ponce
486 et al., 2013). It seems that the disruption of autophagy leads to a differential degradation
487 rate of membrane lipid components, most likely affecting the supply of FAs to sustain
488 other cellular processes, including respiration. Alteration of PE levels was also observed
489 in *atg* mutants under our experimental conditions (Fig. 2). Noteworthy, PE is an
490 essential component of ATG8 conjugation during autophagosome membrane biogenesis
491 (Soto-Burgo et al., 2018; Barros et al., 2020). The less accentuated reductions and
492 accumulation of several PE species (PE 36:4, 36:3(1), 38:2, 36:5, 38:3) in *atg* mutants
493 might be related to the disrupted usage and recycling of these molecules for
494 autophagosome biogenesis, as previously suggested (Avin-Wittenberg et al., 2015;
495 Have et al., 2019).

496 Apart from changes in the extra-plastidial phospholipids, *atg7-2* and *atg5-1*
497 mutants also presented intense degradation of chloroplastic MGDG and DGDG during
498 dark-induced senescence (Fig. 5). This data is consistent with the hypersensitive
499 phenotype marked by chloroplast dismantling observed in *atg* mutants submitted to
500 dark-induced senescence (Thompson et al., 2005; Phillips et al., 2008; Barros et al.,
501 2017). Accordingly, autophagy has been characterized to mediate the degradation of
502 chloroplast proteins by different selective processes (Ishida et al., 2008; Wada et al.,
503 2009; Michaeli et al., 2014; Izumi et al., 2017; Nakamura et al., 2018). This fact aside,
504 the results shown here reinforce the hypothesis that other pathways likely mediate the

505 degradation of chloroplast lipid components in carbon-starved plants and that they are
506 likely to be more active when autophagy is absent. Moreover, this response is not
507 restricted to carbon limitation, as similar results were reported in both Arabidopsis and
508 maize *atg* mutants under low-nitrogen conditions (Supplemental Figure S3)
509 (McLoughlin et al., 2018; Have et al., 2019).

510 The breakdown of membrane lipid components releases FFAs for further
511 oxidation in peroxisomes. The overaccumulation of FFAs is detrimental to the plant
512 cell. Consequently, FFAs are first incorporated as neutral lipids, to then be directed to
513 peroxisomal FA catabolic pathways (Fan et al., 2017). TAGs stored in LDs are not
514 abundant in leaf mesophyll cells. However, their number substantially increases under
515 abiotic conditions and developmental senescence (Pyc et al., 2017; Yang et al., 2018).
516 In agreement with this, we observed a transient LD accumulation after 3d of darkness in
517 WT plants. In addition, a marked increase of highly unsaturated TAG levels was
518 observed after 3d of darkness in WT (Fig. 3). Despite an increase of some FFA species
519 after 3d of darkness, this increase was not overloaded to result in a lipotoxic effect since
520 WT plants present favorable performance under dark-induced senescence (Barros et al.,
521 2017). Indeed, the pattern of initial accumulation and later reduction of FFA 16:2, 18:0,
522 18:2, 18:3 was similar to the observed for TAG and LD (Figs. 4, 5 and 9), suggesting a
523 dynamic equilibrium between membrane consumption, lipid reserve formation, and FA
524 utilization in WT plants. By contrast, not only LD accumulation was disrupted in *atg5-1*
525 and *atg7-2* mutants, but they also showed lower levels of unsaturated TAGs and higher
526 accumulation of plastidial FFA 16:3 after 3d and 6d of darkness. Collectively, these
527 results demonstrate that autophagy is required for the proper mobilization of chloroplast
528 lipids destined for cytosolic LD biosynthesis in Arabidopsis starved cells. A recent
529 study of maize *atg12* mutants subjected to carbon deprivation reported distinct LD
530 dynamics (McLoughlin et al., 2020). It was observed that *atg12* mutants contained more
531 cytoplasmic LD under normal growth conditions. In individually darkened leaves, LD
532 numbers were not affected in WT and only slightly reduced in the *atg12* mutant. A
533 possible explanation for this discrepancy may stem from the experimental systems
534 utilized. While McLoughlin et al. (2020) analyzed individually darkened leaves of
535 maize, we examined Arabidopsis in a whole plant darkening system. Plants have
536 divergent metabolic strategies in response to different darkening treatments (Law et al.,
537 2018). It has been postulated that individually darkened leaves still receive sugars from
538 other parts of the plant during short-term dark periods. However, the lipid catabolic

539 response is not activated as long as carbohydrates are available, which likely affects LD
540 dynamics (Kunz et al., 2009; Law et al., 2018). Not only LD responses diverge
541 according to the darkness treatment imposed, but the results reported until now also
542 open new avenues regarding contrasting LD responses in different plant species.

543 In *Arabidopsis*, autophagy operates in the mobilization of FAs from organelle
544 membranes to TAG synthesis under normal growth conditions (Fan et al., 2019). The
545 same study stated that autophagy has no influence on TAG formation during darkness,
546 but instead operates in LD degradation by lipophagy mechanism. Despite that, general
547 TAG accumulation was observed in *atg* mutants after 6d of darkness, which was not
548 accompanied by cytosolic LD accumulation (Figs 4 and 5). Nonetheless, caution must
549 be taken when comparing the results obtained here with previous findings (Fan et al.,
550 2019) due to the differences of the genotypes analyzed (Barros et al., 2020). Thus,
551 although Fan et al. (2019) demonstrated the existence of lipophagy in different mutant
552 backgrounds, we analyzed plants solely lacking autophagy. When considered together,
553 our results, coupled with the results from Fan and colleagues, highlight the potential
554 role of autophagy as a versatile player in LD metabolism, particularly in starved cells.

555 Beyond the LDs in the cytosol, PG serve as subcompartments for lipid
556 biosynthesis and storage in chloroplasts (Rottet et al., 2015; van Wijk and Kessler,
557 2017). The analysis of PG-related genes indicates a marked activation of PG
558 metabolism in *atg5-1* and *atg7-2* mutants during dark-induced senescence (Fig. 6). In
559 agreement with that, we observed the presence of large PG in chloroplasts of *atg5-1*
560 mutants under extended darkness conditions (Fig. 7A-C). On the other hand, a reduced
561 abundance of the PG core protein FBN1a was observed in *atg* mutants after 6d of
562 darkness (Fig. 7D and Supplemental Figure S2). It is noteworthy that fibrillin proteins,
563 localized on the PG surface, were proposed to play both structural and protection roles
564 in chloroplast, preventing the PG coalescence and delaying thylakoid disintegration
565 (Austin et al., 2006; Ytterberg et al., 2006; Simkin et al., 2007; Singh et al., 2011; Rottet
566 et al., 2015). It is tempting to suggest, therefore, that the reduction of FBN1a protein can
567 be associated with the large size of PG as well as chloroplast sensitive phenotype in *atg*
568 mutants in response to dark-induced senescence. There is a strong correlation between
569 thylakoid dismantling and supersizing of PG during senescence, which is likely
570 associated with the massive deposit of FAs and phytol, released from chloroplast
571 degradation (Lippold et al., 2012; Besagni et al., 2013; Rottet et al., 2015; van Wijk and
572 Kessler, 2017). In light of this, we also verified an increase of *PPH* transcript in *atg5-1*

573 and *atg7-2* mutants compared to WT under darkness (Fig. 6D). It seems reasonable to
574 assume that both the products from the degradation of chlorophyll (Barros et al., 2017)
575 and chloroplast membranes are funnelled for PG formation in *atg* mutants submitted to
576 extended darkness (Fig. 3,7,10). This response can be presumed as a strategic
577 mechanism preventing the accumulation of lipotoxic intermediaries. Collectively, our
578 results imply that the disruption of autophagy triggers a differential response prompting
579 PG rather than cytosolic LD formation under dark-induced senescence. Nonetheless,
580 whether PG can be remobilized to provide lipid-derived energetic substrates remains to
581 be further investigated.

582 It is already known that, during extended darkness, FAs are oxidized by β -
583 oxidation, generating acetyl-CoA that, combined with oxaloacetate, produces citrate to
584 be exported to mitochondria for ATP generation (Kunz et al., 2009). To further
585 understand whether the released membrane lipid components were channeled to the
586 maintenance of the energetic status, we paid particular attention to the transcriptional
587 activation of lipid oxidation pathways. Our results show an up-regulation of
588 peroxisomal genes in plants with disrupted autophagy after 6d and 9d of darkness (Fig.
589 8). Notably, the transcriptional induction of β -oxidation has been correlated to
590 mitochondrial activity in darkened leaves (Law et al., 2018). Interestingly, the same *atg*
591 mutants exhibited higher rates of respiration and an elegant metabolic reprogramming
592 under carbon starvation conditions (Avin-Winttenberg et al., 2015; Barros et al., 2017).

593 Autophagy also plays a role in peroxisome quality control by the selective
594 degradation of damaged peroxisomes, a process known as pexophagy (Marshall and
595 Vierstra, 2018). The accumulation of peroxisome-related transcripts/proteins in *atg*
596 mutants has been reported lately, and it can represent both activation of peroxisomal
597 metabolism or accumulation of oxidized peroxisome (Supplemental Figure S3)
598 (McLoughlin et al., 2018, 2020; Have et al., 2019). Specifically, under carbon starvation
599 conditions, the enrichment of peroxisomal proteins was followed by an increase of
600 glyoxysomal proteins (McLoughlin et al., 2020). This response suggests that
601 peroxisomes are most likely converted to glyoxysomes to sustain high activation of FA
602 β -oxidation pathways during starvation conditions (McLoughlin et al., 2020). The
603 induction of peroxisomal pathways reported here is positively correlated with the high
604 levels of plastidial FFAs in *atg* mutants, which can be interpreted as a response to
605 control FFA levels concomitantly with energy production. When taken together with
606 our work, these studies suggest that the primary metabolism of *atg* mutants is not only

607 rearranged for amino acid catabolism but is also possibly involved in supporting lipid
608 oxidation pathways. In light of this, further studies are clearly required (i) to investigate
609 whether the disruption of LD dynamics and differential membrane recycling of *atg*
610 mutants impact the supply of lipid substrates for FA oxidation pathways and (ii) to
611 characterize the function of autophagy under a range of environmental stresses during
612 this process.

613

614 **Conclusions**

615 In the current work, we present compelling evidence regarding the importance of
616 autophagy to lipid homeostasis in Arabidopsis leaves. During carbon starvation, the
617 products of organellar membrane recycling are channeled to cytosolic LD production.
618 This enables the balance of FA production and turnover. The functional lack of
619 autophagy impacts proper membrane mobilization and activates a general chloroplast
620 lipid degradation program while failing to produce cytosolic LDs. Nonetheless, the
621 products of chloroplast membrane dismantling are possibly channeled to PG generation
622 in *atg* mutants chloroplasts (Fig. 10). We postulate that the increased activation of
623 peroxisomal lipid catabolic pathways is part of a complex metabolic response to
624 catabolize the FFAs released from chloroplast dismantling, and possibly, from PG
625 mobilization. Although our results highlight novel insights regarding the role of
626 autophagy in lipid metabolism, they certainly underestimate the complex crosstalk
627 between autophagy, peroxisome, mitochondria, and the endoplasmic reticulum. An
628 investigation of the interplay involving autophagy, membrane recycling, and organellar
629 maintenance is required to fully understand the implications of autophagy for the
630 regulation of lipid reserve dynamics and energy homeostasis. The combined results
631 presented here are of potential application for the breeding of crops with a higher yield,
632 increased resilience, and it seems reasonable to assume that there is a timely need to
633 accelerate our understanding of the mechanisms controlling autophagy and associated
634 processes in response to environmental conditions.

635

636

Figure 10 - Schematic model of lipid remodeling response of *atg* mutants during extended darkness.

Under extended darkness, the plastidial and extraplastidial membranes, composed by MGDG/DGDG and phospholipids, respectively, are degraded, releasing FAs to be stored as TAGs in LD in the cytosol. LDs and transient FFA donate substrates for β -oxidation in peroxisomes. Meanwhile, in *atg* mutants, there is a substantial degradation of chloroplast lipids, while the production of cytosolic LDs is impaired. Part of the released plastidial FAs, together with phytol derived from chlorophyll degradation, are channelled to the production of PG in the chloroplast. β -oxidation pathways are highly induced to consume overloaded plastidial FFA and, possibly, PG lipids (question mark). Abbreviations: Digalactosyldiacylglycerol (DGDG); Fatty acids (FA); Free Fatty Acids (FFA); Lipid Droplet (LD); Monogalactosyldiacylglycerol (MGDG).

637 Methods

638 Plant Material and Dark Treatment

639 All *Arabidopsis thaliana* (L.) Heynh plants used in this study were of the
 640 Columbia ecotype (Col-0). The T-DNA mutant lines *atg9-4* (SALK_145980) (Shin et
 641 al., 2014), *atg5-1* (SAIL_129B079) (Yoshimoto et al., 2009), *atg7-2* (GK-655B06)
 642 (Hofius et al., 2009) were used in this study. Seeds were surface-sterilized and imbibed
 643 for 4 days at 4°C in the dark on 0.7% (w/v) agar plates containing half-strength
 644 Murashige and Skoog (MS) media (Sigma-Aldrich; pH 5.7). Seeds were subsequently
 645 germinated and grown at 22°C under short-day conditions (8 h light/16 h dark), 60%
 646 relative humidity with 150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. For dark treatments and subsequent
 647 induction of dark-induced senescence, 10- to 14-day-old seedlings were transferred to
 648 soil and then grown at 22°C under short-day conditions (8 h light/16 h dark). Four-
 649 week-old plants were transferred to darkness in the same growth cabinet. Whole rosettes
 650 of two different plants representing an independent replicated by genotype were
 651 harvested at intervals of 0, 3, 6, 9 days after transition to darkness. At least five
 652 biological replicates of each genotype were obtained. They were immediately frozen in
 653 liquid nitrogen and stored at -80°C until further analysis.

654

655

656 **Visualization and Analysis of LDs**

657 To visualize LDs in Arabidopsis leaves, LDs were stained with BODIPY
658 493/503 (Invitrogen; from 4 mg/mL stock in DMSO). The sixth rosette leaf of 4-week-
659 old plants was used for LD visualization and analysis. Leaf discs were stained with
660 BODIPY solution (2µg/mL) in the dark and under vacuum for 10 min. Confocal images
661 were acquired using the FV-1200 confocal microscope (Olympus, Japan) with a
662 60X/1.42 oil immersion objective. Excitation was performed at 488nm, and emission
663 was collected between 500-540nm for BODIPY 493/503 and 650-750nm for the
664 chlorophyll autofluorescence. The LD number was quantified using ImageJ (Schneider
665 et al., 2012). Z-stack series of three images from four plants for each genotype were
666 used for the quantification of LDs.

668 **Lipid Profiling and Free Fatty Acid Analysis**

669 Lipids and natural endogenic free fatty acids were extracted from six biological
670 replicates using the MTBE method described by Salem et al. (2016). Vacuum-dried
671 organic phases were processed using ultra-performance liquid chromatography (on a C8
672 reverse-phase column) coupled with Fourier transform mass spectrometry (exactive
673 mass spectrometer; Thermo Fisher Scientific) in positive and negative ionization modes.
674 The free fatty acid were analysed using ultra-performance liquid chromatography
675 (Waters, Acquity I Class System) coupled with mass spectrometer (Q-Exactive Thermo
676 Fisher Scientific, H-ESI). Processing of chromatograms, peak detection, and integration
677 were performed using REFINER MSH 10 (GeneData). Processing of mass spectrometry
678 data included the removal of the fragmentation information, isotopic peaks, and
679 chemical noise. Selected features were annotated using an in-house lipid database
680 (Lapidot-Cohen et al., 2020). Five-six biological replicates were used for the analysis.

682 **Expression analysis by RT-PCR**

683 Total RNA was isolated using TRIzol reagent (Ambion, Life Technology)
684 according to the manufacturer's recommendations. The total RNA was treated with
685 TURBO DNA-free kit (Invitrogen). The integrity of the RNA was checked on 1% (w/v)
686 agarose gels, and the concentration was measured using a Nanodrop spectrophotometer.
687 Finally, 1 µg of total RNA was reverse transcribed with SensiFAST cDNA Synthesis
688 Kit (Bioline) according to the manufacturer's recommendations. Real-time PCR
689 reactions were performed in a 384-well microlitre using SensiFAST SYBR® No-ROX

Kit (Bioline). The primers used here were designed using the open-source program QuantPrime-qPCR primer designed tool (Arvidsson et al., 2008) and are described in Table S2. The transcript abundance was calculated by the standard curves of each selected gene and normalized using the constitutively expressed genes ACTIN (AT2G37620). Three biological replicates were processed for each experimental condition.

Transmission Electron Microscopy

Small pieces of leaves were cut and fixed in 3% Glutaraldehyde in 0.1M Cacodylate buffer (pH 7.4) 10 hours at room temperature in a desiccator and then transferred to 40°C for the continuation of fixation overnight. The tissues were washed in cacodylate buffer and postfixed and stained with 2% osmium tetroxide, 1.5% potassium ferricyanide in 0.1M cacodylate buffer for 2 hours. Tissues were then washed in cacodylate buffer and dehydrated through a graded series of ethanol treatments for 10 min each step, followed by 100% anhydrous ethanol 3 times, 20 min each, and propylene oxide 2 times, 10 min each. The tissues were infiltrated with increasing concentrations of Agar 100 resin in propylene oxide for 16 hours each step. The tissues were embedded in fresh resin in a 600°C oven for 48 hours. Ultrathin sections, approximately 80 nm thick, were cut on a Leica Reichert Ultracut S microtome, collected onto 200 Mesh carbon-formvar coated copper grids, and stained with Uranyl acetate followed by Reynold's Lead Citrate for 10 min. The sections were examined using Tecnai 12 TEM 100kV (Phillips, Eindhoven, the Netherlands) equipped with MegaView II CCD camera and Analysis® version 3.0 software (SoftImaging System GmbH, Münster, Germany).

Immunoblot Analyses

Plant leaf tissue (~100 mg) was ground in liquid nitrogen, and total proteins were extracted using a protein extraction buffer previously described (Gruis et al., 2002). After the addition of buffer, centrifuged for 15 min at 20,000g; supernatants were collected, and samples were incubated immediately for 10 min at 100°C, vortexed, and samples were adjusted in SDS-PAGE sample buffer (Laemmli, 1970). Total proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (GE Healthcare Life science). Equal loading was validated using Coomassie Brilliant Blue staining of the membrane. For immunodetection, antibodies raised against

724 FBN1a (Agrisera; AS06116) was used in combination with horseradish peroxidase-
725 conjugated goat anti-rabbit antibody (Sigma; ab920)

726 **Statistical analyses**

727 The experiments were conducted in a completely randomized design with 3-6
728 replicates of each genotype. Data were statistically examined using analysis of variance
729 and tested for significant ($P < 0.05$) differences using Student's *t* tests from algorithm
730 embedded into Microsoft Excel. Principal component analysis (PCA) was performed to
731 reduce the dataset and identify the variables that best explained the highest proportion
732 of total variance using Metaboanalyst 4.0, an open-source web-based tool for
733 metabolomics data analysis (Chong et al., 2019).

734

735 **Accession Numbers**

736 The Arabidopsis Genome Initiative locus numbers for the main genes discussed in this
737 article are as follows: *ATG5* (At5g17290), *ATG7* (At5g45900), and *ATG9* (At2g31260).

738

739 **Supplemental Data**

740 The following supplemental materials are available.

741 **Supplemental Figure S1:** Lipid response of *atg* mutants throughout dark-induced
742 senescence. Principal component analysis (PCA) was performed using the lipid data of
743 *atg5-1*, *atg7-2*, *atg9-4* and wild type (WT) genotypes after 0 (0d), 3 (3d), (6d), and 9
744 (9d) days of dark treatment.

745 **Supplemental Figure S2: Quantification of the FBN1a protein signal**

746 Integrated density values for FBN1a were first normalized to loading control and then
747 expressed as a percentage of the obtained value for the wild type (WT) at 0d. Data
748 represent means $\pm$ SE; $n = 3$ biological replicates. An asterisk (*) indicates values that
749 were determined by Student's *t*-test to be significantly different ($P < 0.05$) from the WT
750 at each time point analyzed.

751 **Supplemental Figure S3:** Compared lipidomics of Arabidopsis and maize *atg* mutants
752 under carbon and nitrogen starvation. General representation of the changes in
753 Phospholipids, Galactolipids, Lipid Droplet number, and β -oxidation transcripts reported
754 in Arabidopsis and maize under carbon starvation (this study; McLoughlin et al., 2020)
755 and nitrogen starvation (McLoughlin et al., 2018; Have et al., 2019). The scheme does
756 not take into consideration the experimental differences in these studies. Abbreviations-

757 PC: Phosphatidylcholine; PE-: Phosphatidylethanolamine; PG: phosphatidylglycerol; -
758 C: Carbon starvation; -N: Nitrogen starvation

759 **Supplemental Table S1:** Summary of the main variables of the first three Principal
760 Component (PC) of *atg* mutants and wild type (WT) genotypes after 0 and 6 days of
761 dark treatment. The contribution of which PC is showed as cumulative variance (CV).

762 **Supplemental Table S2:** Primers used in the RT-PCR analyses performed in this study

763 **Supplemental Table S3:** Relative lipid content of leaves of Arabidopsis knockout
764 mutants *atg5-1*, *atg7-2*, *atg9-4*, and wild type (WT) after 0, 3, 6, and 9 days of dark
765 treatment

766 **Supplemental Table S4:** Variables of the first three Principal Component (PC) of *atg*
767 mutants and wild type (WT) genotypes after 0 and 6 days of dark treatment

768

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777

778 FIGURE CAPTIONS

779

780 **Figure 1 - Differential lipid response of *atg* mutants under dark-induced**
781 **senescence.**

782 Principal component analysis (PCA) was performed using the lipid data obtained in
783 samples from 4-week-old Arabidopsis plants immediately before lights were turned off
784 (0 d) and after further treatment for 6d in darkness. Three loss-of-function mutants of
785 the autophagy pathway, namely *atg5-1*, *atg7-2*, *atg9-4* and their respective wild type
786 (WT) were analyzed.

787

788 **Figure 2 - *atg* mutants present altered phospholipid levels under control and**
789 **extended darkness conditions**

790 Hierarchical clustering of (A) Phosphatidylcholine (PC) and (B)
791 Phosphatidylethanolamine (PE). Values plotted are log₂ fold change. Distances were
792 measured by Pearson correlation using MeV 4.9.0 software. Data were normalized to
793 the mean response calculated for the 0-d dark treated leaves of the wild type (WT).
794 Values presented are means ± SE of five-six biological replicates per genotype; an
795 asterisk (*) designates values that were determined by Student's t-test to be significantly
796 different ($P < 0.05$) from WT at each time point analyzed.

797

798 **Figure 3 - Chloroplast lipids are substantially reduced in *atg* mutants during dark-**
799 **induced senescence**

800 Monogalactosyldiacylglycerol (MGDG) and Digalactosyldiacylglycerol (DGDG)
801 values plotted are $\log_2$ fold change. Data were normalized to the mean response
802 calculated for the 0-d dark treated leaves of the wild type (WT). Values presented are
803 means $\pm$ SE of five-six biological replicates per genotype; an asterisk (*) designate
804 values that were determined by the Student's t-test to be significantly different ($P <$
805 0.05) from WT at each time point analyzed.

806

807 **Figure 4 - Distinct accumulation of neutral lipids in *atg* mutants during dark-**
808 **induced senescence**

809 (A) Triacylglycerol (TAG) and (B) Diacylglycerol (DAG). Values plotted are $\log_2$ fold
810 change. Data were normalized to the mean response calculated for the 0-d dark treated
811 leaves of the wild type (WT). Values presented are means $\pm$ SE of five-six biological
812 replicates per genotype; an asterisk (*) designates values that were determined by
813 Student's t-test to be significantly different ($P < 0.05$) from WT each time point
814 analyzed.

815

816 **Figure 5 - Autophagy deficiency affects Lipid Droplet (LD) dynamics during dark-**
817 **induced senescence**

818 (A) Representative confocal images of LDs (BODIPY 493/503 fluorescence, false color
819 (green) in wild type (WT), *atg5-1*, and *atg7-2* leaves. (B) Merged image of BODIPY
820 and chloroplast autofluorescence (false color red). Images were collected at the same
821 magnification and are projections of Z-stacks of 20 optical sections Bar = 10 μ m (C)
822 Number of total LDs per image area. The number of LDs was quantified by ImageJ as
823 BODIPY-stained lipid area in 2D projections of Z-stacks of 3 images of four biological
824 replicates per genotype and time point.

825

826 **Figure 6 - PG related genes are up-regulated in *atg* mutants during dark-induced**
827 **senescence**

828 Transcript abundance is shown for genes associated with plastoglobuli (PG), including
829 PES-1 (A), PES-2 (B), ABC1K7 (C), PPH (D). The y-axis values represent the gene
830 expression level relative to the wild type (WT). Data were normalized with respect to
831 the mean response calculated for the 0-d dark-treated leaves of the WT. Values
832 presented are means $\pm$ SE of three independent biological replicates. An asterisk (*)
833 indicates values that were determined by Student's t-test to be significantly different (P
834 < 0.05) from the WT at each time point analyzed. A number sign (#) indicates values
835 determined by Student's t-test to be significantly different ($P < 0.05$) from WT at 0d.

836

837 **Figure 7: Autophagy disruption triggers altered PG response**

838 (A) Transmission electron micrographs of leaves from 4-week-old Arabidopsis plants
839 immediately before lights were turned off (0 d) and after further treatment for 7 days of
840 darkness. Scale bar = 1 μ m; Quantification of the number (B) and size (C) of
841 plastoglobuli per chloroplasts ($\pm$ SE, $n = 6-7$ chloroplasts of three independent cells);
842 Analysis shown in A-C were performed in *atg5-1* and its respective wild type (WT). (D)
843 Immunoblot analysis of the PG protein, FBN1a, was performed in leaves from 4-week-
844 old Arabidopsis *atg5-1*, *atg7-2* and WT plants immediately before (0 d) and after
845 further treatment for 3d and 6d in darkness. FBN1a protein display a predicted
846 molecular mass to $\sim 30-35$ kD. The detection of a second lower band in the region of

847 30kD possibly corresponds to FBN1b protein (with high sequence similarity with
848 FBN1a) as previously observed in Giacomelli et al., 2006; Matinis et al., 2013; 2014.

849

850 **Figure 8 - Changes in transcript levels in WT and *atg* mutants during dark-**
851 **induced senescence.**

852 Transcript abundance is shown for genes associated with lipid catabolic pathways,
853 including ACX-4 (A), CSY-1(B) KAT-2 (C), MPF-2 (D), pMDH-1 (E), SDP-1 (F). The
854 y-axis values represent the expression level relative to the wild type (WT). Data were
855 normalized with respect to the mean response calculated for the 0-d dark-treated leaves
856 of the WT. Values presented are means $\pm$ SE of three independent biological replicates.
857 An asterisk (*) indicates values that were determined by Student's t-test to be
858 significantly different ($P < 0.05$) from the WT at each time point analyzed. A number
859 sign (#) indicates values determined by Student's t-test to be significantly different ($P <$
860 0.05) from WT at 0d.

861

862 **Figure 9- Changes in free Fatty Acid levels in WT and *atg* mutant plants under**
863 **dark-induced senescence.**

864 Values plotted are $\log_2$ fold change. Data were normalized to the mean response
865 calculated for the 0-d dark treated leaves of the wild-type (WT). Values presented are
866 means $\pm$ SE of five-six biological replicates per genotype; an asterisk (*) designate
867 values that were determined by the Student's t-test to be significantly different ($P <$
868 0.05) from WT each time point analyzed.

869

870 **Figure 10 - Schematic model of lipid remodeling response of *atg* mutants during**
871 **extended darkness.**

872 Under extended darkness, the plastidial and extraplastidial membranes, composed by
873 MGDG/DGDG and phospholipids, respectively, are degraded, releasing FAs to be
874 stored as TAGs in LD in the cytosol. LDs and transient FFA donate substrates for β -
875 oxidation in peroxisomes. Meanwhile, in *atg* mutants, there is a substantial degradation
876 of chloroplast lipids, while the production of cytosolic LDs is impaired. Part of the
877 released plastidial FAs, together with phytol derived from chlorophyll degradation, are
878 channeled to the production of PG in the chloroplast. β -oxidation pathways are highly
879 induced to consume overloaded plastidial FFA and, possibly, PG lipids (question mark).
880 Abbreviations: Digalactosyldiacylglycerol (DGDG); Fatty acids (FA); Free Fatty Acids
881 (FFA); Lipid Droplet (LD); Monogalactosyldiacylglycerol (MGDG).

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