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1 **Respective role of plastoglobules and lipid droplets in leaf neutral lipid accumulation during**
2 **senescence and nitrogen deprivation**

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25

26 **Summary:**

27 Upon abiotic stress or senescence, the size and/or abundance of plastid-localized plastoglobules and
28 cytosolic lipid droplets, both compartments devoted to neutral lipid storage, increase in leaves.
29 Meanwhile, plant lipid metabolism is also perturbed, notably with the degradation of thylakoidal
30 monogalactosyldiacylglycerol (MGDG) and the accumulation of neutral lipids. Although these
31 mechanisms are probably linked, they have never been jointly studied, and the respective roles of
32 plastoglobules and lipid droplets in the plant response to stress are totally unknown. To address this
33 question, we determined and compared the glycerolipid composition of both lipid droplets and
34 plastoglobules, followed their formation in response to nitrogen starvation and studied the kinetics
35 of lipid metabolism in *Arabidopsis* leaves. Our results demonstrated that plastoglobules
36 preferentially store phytyl-esters, while triacylglycerols (TAGs) and steryl-esters accumulated within
37 lipid droplets. Thanks to a pulse chase labeling approach and lipid analyses of *fatty acid desaturase 2*
38 (*fad2*) mutant, we showed that MGDG-derived C18:3 fatty acids were exported to lipid droplets,
39 while MGDG-derived C16:3 fatty acids were stored within plastoglobules. The export of lipids from
40 plastids to lipid droplets was likely facilitated by the physical contact occurring between both
41 organelles, as demonstrated by our electron tomography study. The accumulation of lipid droplets
42 and neutral lipids was transient, suggesting that stress-induced TAGs were remobilized during the
43 plant recovery phase by a mechanism that remains to be explored.

44 **Keywords (5-8):** Plastoglobules, lipid droplets, nitrogen starvation, neutral lipids, chloroplast-lipid
45 droplet contact, senescence

46

47 Introduction

48 Leaf senescence contributes a major part to the relocation of nutrients towards storing tissues, such
49 as roots in the case of perennial plants and seeds in the case of annual plants. A major redistribution
50 of lipids in leaves participates in this process, with a reduction in galactolipid content due to the
51 dismantlement of the plastid membranes, a global decrease of up to 80% of total fatty acid (FA)
52 content and an accumulation of TAGs in senescent leaves (Cohen et al., 2022; Kaup et al., 2002; Lin
53 and Oliver, 2008; Yang and Ohlrogge, 2009). In higher plants, TAGs are mainly stored in seeds, pollen
54 and pericarps of oleaginous fruit (*e.g.*, olives or oil palm fruits) and provide a source of energy and
55 carbon necessary for post-germination growth. TAGs also accumulate in leaves under environmental
56 stress, such as heat stress, drought and nitrogen deprivation (Gaude et al., 2007; Higashi et al., 2015;
57 Lee et al., 2019; Mueller et al., 2015; Mueller et al., 2017). TAGs are stored within specialized
58 cytosolic organelles, lipid droplets (LDs), as well as in plastoglobules within plastids. LDs are formed
59 at the ER membrane by the accumulation of TAGs in a lens within the two ER leaflets. When TAG
60 content reaches a threshold, the TAG lens buds from the ER toward the cytosol; this budding is
61 promoted by various factors such as structural proteins or different lipids (recently reviewed in
62 Henne, 2023). The LD structure is identical in all eukaryote cell types, with a core of neutral lipids
63 delimited by a monolayer of polar lipids derived from the cytosolic leaflet of the ER. The monolayer
64 of seed LDs is constituted by different phospholipids (Tzen et al., 1993), while the neutral core is
65 mainly composed of TAGs and steryl-esters (SEs) (Chapman et al., 2019; Horn et al., 2011; Zhou et al.,
66 2019) but may also contain specific lipids, for example wax esters in jojoba seeds (Miwa, 1971). LDs
67 are also present in other plant organs, such as leaves and roots, but in much lower abundance. LD
68 abundancy in leaves varies during the diurnal cycle (Gidda et al., 2016), with an accumulation of LDs
69 at the end of the dark period, suggesting the origin of these LDs from the recycling of membranes
70 that occurs during the night rather than from *de novo* FA synthesis that takes place during the
71 photosynthetic phase. LDs accumulate in leaves in response to various stress conditions, such as heat
72 and cold stress (Gidda et al., 2016), nitrogen starvation (Brocard et al., 2017; Coulon et al., 2020), and
73 fungus infection (Shimada et al., 2014), but also during natural senescence (Brocard et al., 2017;
74 Shimada et al., 2015; Zhang et al., 2020). Although numerous studies on leaf LDs have been carried
75 out, the exact lipid composition of these LDs, which probably differs from seed LDs, is still unknown.

76 Plastoglobules have a structure similar to LDs, with the stromal leaflet of the thylakoid membrane
77 surrounding the neutral lipid core (Austin et al., 2006). Similar to LDs, they increase in abundance
78 and/or size in response, for example, to high light, drought and nitrogen deprivation, but also during
79 senescence (reviewed in Bréhélin and Kessler, 2008; van Wijk and Kessler, 2017). The lipid
80 composition of plastoglobules is not clearly established and seems to vary according to the type of

81 plastid or growth conditions. With regard to the polar lipid monolayer, the identity and relative
82 content of phospholipids are unclear (Deruère et al., 1994; Hansmann and Sitte, 1982; Lichtenthaler,
83 1968; Tevini and Steinmüller, 1985; Wu et al., 1997). Several studies have described the presence of
84 galactolipids in plastoglobules (Greenwood et al., 1963; Hansmann and Sitte, 1982; Hernández-
85 Pinzón et al., 1999; Leggett Bailey and Whyborn, 1963; Lichtenthaler, 1968). However, some authors
86 have suggested that galactolipids can result from contamination by thylakoid membranes (Tevini and
87 Steinmüller, 1985; Tevini and Steinmüller, 1985), and their actual presence in the plastoglobules
88 remains to be firmly confirmed. TAGs are reported to be abundant constituents of plastoglobules in
89 various plastids, such as spinach chloroplasts (Steinmüller and Tevini, 1985), *Viola* and *Sarotamnus*
90 chromoplasts (Hansmann and Sitte, 1982; Steinmüller and Tevini, 1985), and wheat etioplasts (Dahlin
91 and Ryberg, 1986), with a sharp decrease of TAG content during senescence in the plastoglobules of
92 *Fagus sylvatica* leaves (Tevini and Steinmüller, 1985). It is now well established that the neutral lipid
93 core of plastoglobules also contains prenyl quinones, such as plastoquinone, tocopherols
94 (Greenwood et al., 1963; Leggett Bailey and Whyborn, 1963; Lichtenthaler, 1968; Steinmüller and
95 Tevini, 1985; Vidi et al., 2006; Zbierzak et al., 2009), and phyloquinone (Lohmann et al., 2006), as
96 well as fatty acid phytyl-esters (Gaude et al., 2007; Lippold et al., 2012). Chromoplast plastoglobules
97 also store various carotenoids and carotenoid esters (Morelli et al., 2023; reviewed in van Wijk and
98 Kessler, 2017; Zita et al., 2022), which are responsible for fruit color.

99 The decrease in FA synthesis and galactolipid content, concomitant with an increase in TAG and
100 phytyl-ester content in leaves, in addition to the presence of larger and/or more abundant
101 plastoglobules and the appearance of newly formed LDs, during senescence or under stress
102 conditions, suggest a role for both plastoglobules and LDs as a buffer reservoir for FAs resulting from
103 membrane degradation (Ischebeck et al., 2020; Rottet et al., 2015; reviewed in Yu et al., 2021).
104 However, the respective roles of LDs and plastoglobules in the storage of neutral lipids have never
105 been explored in parallel. TAGs are assembled at the ER through two main pathways. In the Kennedy
106 pathway, two acyltransferases, the GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE (GPAT) and the
107 LYSO-PHOSPHATIDIC ACID ACYLTRANSFERASE (LPAT), sequentially esterify two acyl-CoAs to the
108 glycerol 3-phosphate to produce phosphatidic acid (PA), which is then dephosphorylated by the
109 PHOSPHATIDIC ACID PHOSPHATASE (PAP) to give diacylglycerol (DAG). The DIACYLGLYCEROL
110 ACYLTRANSFERASE (DGAT) subsequently catalyzes the acylation of DAG to TAG. The second pathway
111 does not use acyl-CoA as acyl donor but involves the PHOSPHOLIPID:DIACYLGLYCEROL
112 ACYLTRANSFERASE (PDAT) which catalyzed the transfer of an acyl group from the
113 phosphatidylcholine to DAG (reviewed in Xu and Shanklin, 2016). In addition, some TAG synthesis is
114 also believed to occur in the plastid (Kaup et al., 2002; Lippold et al., 2012). The synthesis of SEs is

115 catalyzed either by an ACYL-COA:STEROL ACYLTRANSFERASE (ASAT) or a PHOSPHOLIPID:STEROL
116 ACYLTRANSFERASE (PSAT) depending on the acyl donor substrate (Reviewed in Ferrer et al., 2017). In
117 Arabidopsis, phytol-ester synthesis is catalyzed by PHYTYL-ESTER SYNTHASES PES1 and PES2 (Lippold
118 et al., 2012) that are located at the plastoglobules (Vidi et al., 2006; Ytterberg et al., 2006).TAG,
119 steryl-, and phytol-ester biosynthesis are thought to play a protective role against cytotoxic lipid
120 intermediates (*e.g.*, free FAs (FFAs) and phytol) formed during membrane degradation or lipid
121 remodeling (Bouvier-Navé et al., 2010; Fan et al., 2017; Lu et al., 2020). In addition, neutral lipids
122 accumulated in response to stress conditions probably play important roles during the post-stress
123 recovery phase by providing FAs to facilitate membrane reconstruction or for energy supply through
124 the β -oxidation pathway (Yu et al., 2021; reviewed in Zienkiewicz and Zienkiewicz, 2020).

125 To gain insight into the respective importance of plastoglobules and LDs under conditions that induce
126 thylakoid dismantlement and neutral lipid storage, we monitored the lipid composition of
127 Arabidopsis leaves during natural senescence, senescence artificially induced by nitrogen starvation
128 and reversion after nitrogen supplementation. We compared the lipid composition of both
129 compartments and established that TAGs were mostly stored in LDs, while plastoglobules were
130 dedicated to the storage of phytol-esters. We showed that during senescence, FAs derived from the
131 degradation of MGDG segregated between the plastoglobules and the LDs, with C16:3 FAs remaining
132 in the plastids, while C18:3 FAs were exported to LDs, probably through the contact sites between
133 plastids and LDs, as shown by electron tomography.

134

135 **Material and methods**

136 **Plant material and culture conditions:**

137 *Arabidopsis thaliana* ecotype Columbia (Col-0) were grown as described in (Brocard et al., 2017) at
138 high density on soil in long day conditions (16h light /8h dark), 100 μmol of $\text{photon}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at 20-22°C
139 and 65% humidity.

140 For nitrogen starvation assays, sterile *A. thaliana* seeds were sown on half strength Murashige and
141 Skoog (MS½) medium and grown in long day conditions, at 20–22°C, 16h/8h light/dark cycle for two
142 weeks. Then plantlets were transferred on synthetic minimal medium deprived of nitrogen [0.8%
143 agarose, 1% sucrose, 1mM MgSO_4 , 1mM KH_2PO_4 , 25 μM Fe-EDTA, 35 μM H_3BO_3 , 7 μM MnCl_2 , 0.25 μM
144 CuSO_4 , 0.5 μM ZnSO_4 , 0.1 μM Na_2MoO_4 , 5 μM NaCl, 5nM CoCl_2 , 1mM CaCl_2 and 2.5mM KCl] (Gaude et
145 al., 2007), grown for 10 days more, and transferred back to MS½ plates to recover for 10 days. Note
146 that control plantlets were grown simultaneously and similarly transplanted but in new MS½ plates.

147

148 **Lipid analyses**

149 For analysis of plant extracts, total lipids from aerial parts were extracted by Folch method (Folch et
150 al., 1957) after phospholipase D inactivation using hot isopropanol, as described in Coulon et al.
151 (2020). For LD and plastoglobule lipid analyses, neutral and polar lipids from the total lipid extract
152 were separated by liquid/liquid extraction as described in Coulon and Bréhélin (2021). The different
153 lipid classes are then separated by thin layer chromatography (TLC). For neutral lipid analyses of
154 nitrogen starved plantlets, plates were developed in hexane:diethyl ether:acetic acid (45:7.5:1, v/v/v)
155 according to Juguelin et al. (1986). For neutral lipid analyses of LDs or plastoglobules, and of
156 senescent leaves, plates were developed firstly in Toluene to a height of 10 cm, then in
157 hexane:diethyl ether:acetic acid (45:7.5:1, v/v/v) to a height of 5 cm (Coulon et al., 2020) to allow the
158 separation of phytyl-esters from steryl-esters. Polar lipids were separated with a Vitiello-Zanetta
159 solvent mixture (Vitiello and Zanetta, 1978). The lipids were visualized with primuline under UV light.
160 After FA trans-esterification, lipids were quantified by gas chromatography (Agilent 7890 gas
161 chromatograph equipped with a Carbowax column Alltech Associates, Deerfield, IL, USA) coupled to
162 a flame ionization detector (FID) as described in (Coulon et al., 2020). The retention times of FA-
163 methyl esters were determined by comparison with standards, and were quantified using
164 heptadecanoic acid methyl ester as standard.

165

166 **Estimation of the lipid droplet composition:**

167 Analysis of the purified LD fraction revealed the presence of phytyl-esters (from 17,9 to 37,6 %mol,
168 depending to the LD preparation), which we assumed to be solely the result of contamination by
169 plastoglobules. Consequently, the LD fraction is also contaminated by the MAGs, DAGs, FFAs, TAGs
170 and SEs contained in plastoglobules. In plastoglobules, phytyl-esters represent 75.2% on average of
171 the neutral lipids. Knowing the relative composition of the different other neutral lipids in
172 plastoglobules, and the amount of phytyl-esters detected in LDs, we then calculated the content (in
173 moles) of each contaminating lipids in each LD fraction (n=5), with the formula:

$$\text{contaminating lipid in LD fraction\#N(mol)} = \frac{\text{average \% of the considered lipid in plastoglobules} \times \text{FAPes in LD fraction\#N (mol)}}{\text{average \% of FAPes in plastoglobules}}$$

174 The neutral lipid content of the LDs was then calculated by subtracting the content of each
175 contaminating neutral lipid in LDs to the initial values. Finally, the molar distribution of LD neutral
176 lipids, the mean and the standard deviation were calculated.

177

178

179 **Liquid chromatography – Mass spectrometry (LC-MS/MS) analyses**

180 For the analysis of galactolipids and steryl-esters by LC-MS/MS, internal standards (17:0 cholesteryl
181 ester and 17:0/14:1 phosphatidylcholine (PC) from Avanti Polar Lipids) were added to lipid extract
182 and LC-MS/MS (multiple reaction monitoring mode) analyses were performed with a QTRAP 6500
183 (ABSciex) mass spectrometer coupled to a LC system (1290 Infinity II, Agilent) in positive ion mode.
184 The areas of LC peaks were determined using MultiQuant software (version 2.1; ABSciex) for relative
185 lipid quantification.

186 For the analysis of phospholipids by LC-MS/MS, internal standard (18:0-18:0
187 phosphatidylethanolamine (PE) and 16:0-18:0 sulfoquinovosyldiacylglycerol (SQDG) extracted from
188 spinach thylakoid (Demé et al., 2014) and hydrogenated as described in Buseman et al (2006)) were
189 added to lipid extract. LC-MS/MS (multiple reaction monitoring mode) analyses were performed in
190 positive ion mode with a 6460 triple quadrupole mass spectrometer (Agilent) equipped with a Jet
191 stream electrospray ion source and coupled to an Agilent 1200 HPLC system. Mass spectra were
192 processed by MassHunter Workstation software (Agilent) for identification and quantification of
193 lipids. Lipid amounts (pmol) were corrected for response differences between internal standards and
194 endogenous lipids and by comparison with a quality control (QC). QC extract correspond to a known
195 lipid extract from Arabidopsis cell culture qualified and quantified by TLC and GC-FID as described by
196 Jouhet et al. (2017). For a complete description of the LC-MS/MS methods used in this paper, see
197 Methods S1.

198 **Transmission electron microscopy (TEM) imagery and Tomography**

199 Chemical fixation of leaves was performed as described in Brocard et al. (2017). Briefly, leaves were
200 fixed in 2.5% Glutaraldehyde, post-fixed by 1% OsO₄ and embedded in SPURR (Electron Microscopy
201 Science, EMS) resin. Ultrathin sections of 80 nm thickness were obtained with ultramicrotome LEICA
202 EM UC7. Transmission electron microscope FEI Tecnai G2 Spirit TWIN 120kV equipped with a CCD
203 16Mpixels Eagle 4k camera was used for acquisition (120kV). Image analysis was performed with
204 ImageJ software (Schneider et al., 2012). For electron tomography, FEI software was used for tilt
205 series acquisition, Alignment and backprojection steps were respectively achieved with IMOD and
206 tomoJ softwares, and OSART algorithm with 100 iterations was used.

207 **LD and plastoglobules purification**

208 LD and plastoglobules were purified from 6-week-old Arabidopsis leaves by floatation on sucrose
209 gradients, as described in Brocard et al. (2017) and Coulon and Bréhélin (2021) respectively. Briefly,
210 for LD purification leaves were homogenized in cold G-20 buffer (100 mM Tricine (pH7.5), 10 mM
211 KCl, 1 mM EDTA, 1 mM PMSF, plant protease inhibitor cocktail (Sigma), and 20% sucrose), filtered
212 through two layers of cheese cloth and one layer of Miracloth (Calbiochem) and centrifuged at
213 100 000 g for 30 min. The floating lipid pad was collected and resuspended in 10 mL G-20 buffer
214 using a Potter homogenizer, covered with G-15 buffer (G-buffer with 15% sucrose) and G-5 buffer (G
215 buffer with 5 % sucrose), and centrifuged 50 min at 150 000 g. This step was repeated once. Finally,
216 the floating lipid pad was resuspended in 5 mL G-20 buffer with a Potter homogenizer, covered with
217 G-0 buffer (G buffer without sucrose), and centrifuged 50 min at 150 000 g. The final floating lipid
218 pad constituted the enriched LD fraction, and was stored at -80°C until further analyses. For
219 plastoglobule purification, leaves were homogenized in HB buffer (450 mM sorbitol, 20 mM Tricine-
220 KOH pH 8.4, 10 mM EDTA, 10 mM NaHCO₃, 1 mM MnCl₂, 5 mM Na-ascorbate, 0.05% (w/v) bovine
221 serum albumin, 1 mM PMSF, plant protease inhibitor cocktail), filtered as for LD purification and
222 centrifuged for 8 min at 1,000 g. Pelleted chloroplasts were resuspended in TrE buffer (5 mM Tricine-
223 KOH pH 7.5, 0.2 mM EDTA, 0.2 mM DTT) and centrifuged for 10 min at 2,700 g. The chloroplast pellet
224 was resuspended in 0.2 M sucrose/TrE, homogenized in a 50 mL Potter and centrifuged for 1h at
225 100,000g. The pellet of plastid membranes was resuspended in 45% sucrose/TrE buffer, poured on a
226 gradient of 38 %, 20%, 15% and 5% sucrose, and centrifuged overnight at 100,000 g. The floating
227 plastoglobules are homogenized in 45% sucrose/TrE buffer and finally washed on a gradient of 15%,
228 5% and 0% sucrose.

229 **Protein analyses**

230 For immunoblot experiments, proteins extracted according to Rensink et al. (1998) and concentrated
231 by chloroforme-methanol precipitation (Wessel and Flugge, 1984), were separated by SDS-PAGE and
232 blotted onto nitrocellulose membrane. Blots were probed with sera raised against plastoglobule, ER,
233 plastid envelope or thylakoid markers, respectively AtFBN1b (Vidi et al., 2006), BiP (Höfte and
234 Chrispeels, 1992), AtToc75 (Hiltbrunner et al., 2001), or P16 (Vallon et al., 1986). Proteins were
235 quantified either according to the Bradford method (Bradford, 1976) or the QuantiPro BCA assay kit
236 manual (Sigma).

237 **Preparation of anti-AtLDAP1 serum**

238 ORFs clones of *AtLDAP1* (U17438) in *pENTR* vector were obtained from ABRC center and verified by
239 sequencing. AtLDAP1 ORF was then transferred by GATEWAY® recombinational cloning technology in
240 frame with the included 6xHistidine tag of the pHGWA vector (Busso et al., 2005). The full-length
241 protein was expressed in *Escherichia coli* BL21 (DE3) (Novagen) and purified under denaturing
242 conditions by nickel nitrilotriacetic acid affinity chromatography (Qiagen, Basel, Switzerland)
243 according to the manufacturer's recommendations. Polyclonal antibodies were produced in rabbit
244 (Covalab, Villeurbanne, France).

245

246 **Radiolabeling**

247 Two-week-old plantlets grown on MS½ medium were pulse-labeled by incubation with 7 mL MS½
248 medium supplemented with [¹⁴C] acetate (20 µCi) for 2h. Then, plantlets were rinsed three times in 7
249 mL MS½ medium to remove the excess of [¹⁴C] acetate. The chase was done by transferring plantlets
250 either on synthetic minimal medium depleted of nitrogen or on MS½ medium for 10 days. After lipid
251 extraction and separation by TLC, the radioactivity incorporated into neutral and polar lipids was
252 quantified using a Typhoon FLA9500 (GE Healthcare).

253

254 **Results:**

255 **During *Arabidopsis* leaf ageing neutral lipid content increases and both plastoglobules and LDs**
256 **accumulate**

257 To monitor the lipid composition of *Arabidopsis* leaves in parallel with the accumulation of
258 plastoglobules and LDs, we grew *Arabidopsis* plantlets for 6 weeks on soil at high density. At the end
259 of these 6 weeks, the plantlets entered senescence but also underwent stress conditions, probably
260 due to the high density, as shown by the purple color of some leaves (Fig. 1A). Ultrastructural study
261 of these 6-week-old plantlets showed that the leaves were at different stages of senescence, with
262 more or less pronounced dismantling of the thylakoid membranes (Fig. 1B). An average of only 0.02
263 LD per cross section of parenchyma cells was observed in parenchyma cells of 3-week-old leaves
264 whereas 6-week-old green leaves had an average of 1.27 LD per cross section of parenchyma cell and
265 an LD mean diameter of 817 (± 251) nm (Fig. 1C). In parallel, the size of plastoglobules increased
266 considerably from a mean diameter of 70 (± 14) nm in 3-week-old leaves to 226 (± 59) nm in the most
267 senescent 6-week-old leaves. The polar and neutral lipid composition showed a decrease in MGDG
268 and phosphatidylglycerol (PG)/PE content in the 6-week-old plantlets compared to the 4-week-old
269 plantlets, in parallel with an accumulation of neutral lipids, mainly TAGs, steryl- and phytol-esters
270 with a 28-, 6- and 18-fold increase, respectively (Fig. 1D). Unlike MGDG, the digalactosyldiacylglycerol
271 (DGDG) content was not significantly affected. The compositions of MGDG and DGDG were stable
272 during leaf aging (Figs. 2 and S1), while a decrease in relative C16:0 content was observed in TAGs
273 and phytol-esters, in favor of an increase in relative C16:3 content (from 61% to 72%) for phytol-
274 esters and C18:3 (from 43% to 61%) for TAGs. A slight increase in relative C18:3 content (from 32% to
275 36%) in SEs was also observed, concomitant with a decrease in C18:2 content (from 38% to 26%).
276 These data suggest that TAGs, steryl- and phytol-esters, which accumulated in senescing leaves, are
277 formed mainly by esterification of MGDG-derived C16:3 and C18:3 FAs, as previously suggested
278 during freezing or heat stress (Moellering et al., 2010; Mueller et al., 2017).

279 **Plastoglobules from 6-week-old leaves accumulate mostly phytol-esters and contain a limited**
280 **amount of TAGs and SEs**

281 To determine which neutral lipids accumulate during senescence in plastoglobules and LDs, we
282 purified both compartments from 6-week-old *Arabidopsis* leaves and determined their glycerolipid
283 composition. The fraction purity was assessed by immunoblot (Fig. 3A) with Golgi, ER, plastid
284 membranes and plastoglobule markers. To obtain an LD marker, we raised serum against *Arabidopsis*
285 LIPID DROPLET ASSOCIATED PROTEIN1 (AtLDAP1), a protein specific to leaf LDs (Brocard et al., 2017;
286 Gidda et al., 2016). As shown in Fig. 3A, the plastoglobule fraction was free of any membrane or LD

287 contaminant. We then determined the neutral lipid composition of the plastoglobules by TLC/GC-FID
288 (Fig. 3B). Surprisingly, the neutral lipids in plastoglobules of 6-week-old leaves contained less than 2
289 mol% of TAGs but were mainly composed of phytol-esters (75 mol%), with SEs (7 mol%), some FFAs
290 (7 mol%), MAG (7 mol%) and DAG (2 mol%). The TAGs of the plastoglobules (Fig. 4A) had a different
291 composition to that of the total leaves (Fig. 2B), with only 10% and 18% C18:2 and C18:3,
292 respectively, in the plastoglobules compared with 21% and 43% in the leaves, but an enrichment in
293 C16:0, C16:2, C16:3, C18:0 and C18:1. The presence of phytol-esters in plastoglobules was expected,
294 as previously reported by Gaude et al. (2007). Their FA composition (Fig. 4B) was similar to that of
295 Gaude's study, with a large majority of C16:3. The presence of SEs in plastoglobules was more
296 surprising, as SEs are mainly found in mitochondrial and nuclear membranes, microsomes, and
297 cytosolic LDs (Ferrer et al., 2017). However, their FA composition was different between
298 plastoglobules and LDs, with enrichment of C14:0 and C16:3 in plastoglobules and C18:3 in LDs. To
299 confirm the identity of SEs in plastoglobules, we performed a detailed lipidomic profiling of SEs using
300 LC-MS on lipid extracts from plastoglobules. The LC-MS data confirmed the genuine presence of
301 specific SEs in plastoglobules, such as C14:0-sitosterol and C16:2-sitosterol, and an enrichment of
302 C16:3 SEs at the expense of C18:3 SEs (Fig. 4D). It should be noted that the SE acyl distribution
303 determined by LC-MS differed from that obtained by GC-FID. In particular, saturated C16:0- and
304 C18:0-SEs were not found by LC-MS analysis while detected in significant amounts by GC-FID. LC-MS
305 quantification depends on the ionization yield of the studied molecule, which varies depending on
306 the nature of the molecule (Jouhet et al., 2017). We compared the detection of C18:0-, C18:1-, and
307 C18:2-cholesterol ester standards by LC-MS and observed that the ionization yield of saturated
308 C18:0-cholesterol ester is 10 times lower than that of C18:2-cholesterol ester (Fig. S2), while the
309 amount of each standard was similar according to the quantification performed by GC-FID. Such
310 underestimation by LC-MS of saturated cholesterol esters certainly also applies for other SEs and
311 explains the difference of SE composition observed between LC-MS and GC-FID.

312 MGDGs, DGDGs and PGs were detected in plastoglobules, but their quantity was below the threshold
313 to be quantified by GC-FID. Therefore, we determined by LC-MS the distribution of the molecular
314 species in each polar lipid class. While the proportion of each molecular species constituting DGDG in
315 the plastoglobules (Fig. 5A) was similar to that in the thylakoids, the proportion of molecular species
316 of MGDG was significantly different (Fig. 5B), with a particular enrichment of MGDG 36:6 in the
317 plastoglobules (49% in the plastoglobules and 38% in the thylakoids). Furthermore, according to our
318 LC-MS measurements, the plastoglobule MGDG/DGDG ratio equaled 3:1, whereas it was around 1:1
319 in the thylakoids (Fig. 5C). These results suggest that galactolipids are not contaminants but genuine
320 components of the plastoglobule monolayer. The MGDG/DGDG ratio of 1 obtained for thylakoids by
321 LC-MS in this study does not correspond to an absolute quantification of MGDG and DGDG, and is

322 given as an indication for comparison between the different analyzed fractions. Indeed, as explained
323 above, the ionization yield differs depending on the nature of the molecule analyzed and an absolute
324 quantification by LC-MS is not possible. This explains the difference with the value of the
325 MGDG/DGDG ratio, which is generally estimated between 2 and 3 for Arabidopsis leaves (Dormann
326 et al., 1995; Jarvis et al., 2000; Yu et al., 2020). The composition of the phospholipids present in the
327 plastoglobules was also determined by LC-MS, and a slight enrichment in saturated FAs in the
328 plastoglobule PG was observed compared with those of the thylakoids (Fig. S3).

329

330 **LDs are mostly comprised of TAGs and steryl-esters**

331 The immunoblots we performed on the LD-enriched fraction (Fig. 3A) showed that it also contained
332 plastoglobules, as noted in our previous proteomic study (Brocard et al., 2017). This contamination
333 originates from the inevitable chloroplast breakage that occurs during LD purification, releasing some
334 plastoglobules. Nevertheless, we established the glycerolipid composition of this LD-enriched
335 fraction (Fig. 3C and D) in a similar way to that achieved with the plastoglobules and were able to
336 determine the proportion of plastoglobule contamination in the LD lipidome (see below).

337 In contrast to plastoglobules, the neutral lipids in the LD-enriched fraction consisted mainly of SEs
338 and TAGs (Fig. 3C), but phytol-esters accounted for 29%mol of the total lipid content. Phytol-esters
339 are wax esters containing phytol and are located in the plastids, particularly in the plastoglobules
340 (Gaude et al., 2007). Thus, we hypothesized that phytol-esters in the LD-enriched fraction originated
341 from contamination by plastoglobules. This was confirmed by the FA composition of the phytol-
342 esters, which was similar to that of the plastoglobule phytol-esters (Fig. 4B). With this premise and
343 our analysis of the plastoglobules showing that phytol-esters represented 75%mol of the total lipid
344 content of the plastoglobules, we recalculated the neutral lipid composition of the LD-enriched
345 fraction by excluding phytol-esters and the proportion of other individual neutral lipids originating
346 from contamination by the plastoglobules. The proportion of TAGs and SEs in the LDs was then
347 estimated at 26 and 65%mol, respectively (Fig. 3D). The FA composition of TAGs in LDs (Fig. 4B) was
348 similar to the composition of TAGs in 6-week-old leaves (Fig. 2B) and was totally different from TAGs
349 in plastoglobules, with, for example, a large majority of C18:3 (58%) and C18:2 (18%) in LDs
350 compared with 18 and 10%, respectively, in plastoglobules. These results imply that most of the leaf
351 TAGs are stored in LDs and that the small amount of TAGs found in the plastoglobules, with their
352 specific FA composition, probably originates from a different biosynthetic pathway.

353 Few quantities of MGDG and DGDG were detected in the LD-enriched fraction. The distributions of
354 their molecular species were determined by LC-MS and found to be significantly different from those
355 of plastoglobules and thylakoids (Fig. 5), with enrichment in MGDG 34:6 (57, 32 and 44% in LDs,
356 plastoglobules and thylakoids, respectively) and DGDG 34:3 (37, 23 and 20% in LDs, plastoglobules

357 and thylakoids, respectively) and depletion in MGDG 36:6 (23, 49 and 38% in LDs, plastoglobules and
358 thylakoids, respectively) and DGDG 36:6 (24, 53 and 51% in LDs, plastoglobules and thylakoids,
359 respectively). The presence of galactolipids in extra-plastidial LDs was surprising, but their specific FA
360 compositions suggest that they were not derived from contamination by plastoglobules or thylakoids
361 but rather were true constituents of LDs. This suggests an export of lipids across the plastidial
362 envelope to the LDs and possibly other extraplastidial compartments, as previously described during
363 phosphate starvation (Härtel et al., 2000; Klaus et al., 2002).

364 More generally, the overall FA compositions of neutral lipids differed in plastoglobules and LDs (Fig.
365 4E), with the majority of C16:3 (56%) in plastoglobules, whereas neutral lipids in LDs were mainly
366 composed of C18:3 (49%) and C18:2 (20%), suggesting that the neutral lipids contained in these two
367 compartments did not have the same origin.

368

369 **MGDG-derived FAs are incorporated into TAG and phytyl-/steryl-esters during nitrogen starvation**

370 To determine the origin of the neutral lipids accumulated in plastoglobules and LDs during
371 senescence, we induced artificial senescence by growing Arabidopsis seedlings on nitrogen-deprived
372 medium (Fig. 6A) and monitored glycerolipid metabolism by radioactive labeling.

373 For the pulse-chase experiment, 2-week-old Arabidopsis seedlings were first fed with radioactive
374 [¹⁴C]acetate for 2 hours and then nitrogen starved for 10 days. Prior to radioactivity quantification,
375 lipids were separated by TLC using a protocol that did not allow phytyl-esters to be separated from
376 SEs. All polar glycerolipids except DGDGs, which are mainly synthesized after an import of lipids from
377 the ER (Browse et al., 1986), were initially labeled, with most of the radioactivity incorporated into
378 MGDG, PC and PG (42, 27 and 13%, respectively; Fig. 6G and Table S1). In contrast, neutral lipids
379 were barely radiolabeled, with only 1.3, 0.4 and 0.2% of the total radioactivity in FFAs, TAGs and
380 phytyl-/steryl-esters, respectively (Table S1).

381 After 10 days of nitrogen starvation, MGDG radiolabeling decreased from 42% to 35% of total
382 radioactivity, while it remained stable in the seedlings grown on a nitrogen-balanced medium. These
383 results reflect the remobilization of MGDG during nitrogen starvation. The incorporation of the
384 radiolabel into DGDG over the 10-day period was similar in both starved and well-fed plantlets,
385 demonstrating that this incorporation was not linked to induced senescence but rather derived from
386 standard DGDG metabolism, which was apparently unaffected by starvation. At the same time,
387 radioactivity was incorporated into TAGs and phytyl-/steryl-esters at a higher rate in starved plantlets
388 than in supplemented ones (8.9% and 9.3% incorporation of the total radioactivity in starved
389 plantlets versus 0.3% and 5.3% in control plantlets, in TAGs and phytyl-/steryl-esters, respectively,
390 Fig. 6G). This confirmed that TAGs and phytyl-/steryl-esters were synthesized in response to nitrogen
391 starvation but also demonstrated that their synthesis involved, at least in part, the incorporation of

392 FAs previously constituting other lipids rather than *de novo* synthesized FAs. MGDG is the lipid
393 showing the fastest and greatest loss of radioactive labeling, while the labeling of other polar lipids is
394 more or less similar between starved and supplemented plantlets (Fig. 6G and Table S1). This
395 indicates the incorporation of MGDG-derived FAs into TAGs and phytyl-/steryl-esters during
396 senescence. In addition, during MGDG degradation, no increase in the radiolabel of FFAs was
397 observed, with a remarkably low level of radiolabeled FFAs throughout the kinetics (Table S1),
398 suggesting that no MGDG-derived FAs accumulated as FFAs in the leaf.

399 In parallel, the glycerolipid distribution and composition of the nitrogen-starved plantlets were also
400 determined (Fig. 7). As observed in naturally aging plants, the relative MGDG content in N-starved
401 plantlets decreased considerably, but its FA composition remained stable and comparable between
402 the starved and control plantlets (Fig. S4). At the same time, the relative DGDG content increased
403 during starvation, while the relative phospholipid content was stable and similar in the starved and
404 control plantlets. The TAG and phytyl-/steryl-ester content increased significantly in response to
405 nitrogen deprivation, with an accumulation of C16:3 in phytyl-/steryl-esters and C18:3 in TAGs, both
406 at the expense of saturated FAs. Overall, these results indicate that, during natural or artificially
407 induced senescence, MGDG degradation releases C16:3 and C18:3 FAs, which are incorporated into
408 neutral lipids accumulating in LDs and plastoglobules.

409
410 In agreement with these results, TEM observations revealed that after 10 days of nitrogen starvation,
411 the cross-sectional area of the plastoglobules increased from *ca.* 0.003 μm^2 (or 60 nm in diameter) to
412 *ca.* 0.034 μm^2 (210 nm in diameter). In addition, cytosolic LDs were not observed in parenchyma cells
413 before the starvation period (based on the observation of 40 cells) but accumulated to reach a
414 maximum of 0.013 LD per μm^2 of cell surface with an average LD cross-sectional area of 0.63 μm^2
415 (Fig. 6B–E). As described in Gaude et al. (2007), the chloroplast ultrastructure was seriously
416 disrupted, with disorganized grana and a dramatic accumulation of starch granules distorting the
417 thylakoid network.

418
419 To further elucidate the origin of FAs used for neutral lipid synthesis, the FA composition of TAGs and
420 phytyl-/steryl-esters was determined in *fad2* mutant seedlings before and after 10 days of nitrogen
421 starvation. *fad2* mutant is deficient in the ER-localized desaturase that is responsible for the C18:1 to
422 C18:2 desaturation of ER phospholipids, and displays reduced amount of C18:3 and C18:2 but
423 increased amount of C18:1 mainly in extraplastidic polar lipids, compared to the wild-type (Miquel
424 and Browse, 1992, and Fig. S5). Phytyl-/steryl-esters in *fad2* mutant were enriched in C18:1 and
425 impoverished in C18:2 compared to wild-type, both before and after nitrogen starvation (Fig. 8A),
426 but contained C18:3 in amount similar to wild-type (49 vs 43% before starvation and 45 vs 41 % after

427 nitrogen starvation in *fad2* and *Col0* respectively), suggesting that both ER- and plastid-derived FAs
428 contribute to phytyl-/steryl-ester synthesis.

429 Before nitrogen starvation, TAGs in *fad2* mutant contained 5 times more C18:1 levels than wild type
430 (Fig. 8B), but no C18:2 and very few C18:3 (less than 4% of total FAs), suggesting that FAs
431 contributing to basal TAG synthesis are mainly ER-derived. After 10 days of nitrogen starvation, C18:1
432 content decreased both in wild type and *fad2* TAGs, but was still 10 times higher in *fad2* mutant than
433 in wild-type. In parallel, C18:3 content increased both in wild-type and *fad2* TAGs after nitrogen
434 starvation to reach 59 and 49% of fatty acids, respectively. This indicates that some FAs desaturated
435 in PCs by FAD2 are incorporated under nitrogen starvation into TAGs, but that the C18:3 present in
436 starvation-induced TAGs mainly derived from chloroplastic lipids.

437

438 **Neutral lipids are remobilized during repletion**

439 The fate of neutral lipids accumulated in leaf plastoglobules and LDs is still unclear. They could
440 represent a stock of energy, as described in seeds or a pool of FAs directly available for *de novo*
441 membrane biogenesis or membrane remodeling when environmental conditions improve, as
442 described for pollen tube growth (Hernández et al., 2020; Ischebeck, 2016; Mellema et al., 2002). To
443 address this point, nitrogen-starved plantlets were transferred back to nitrogen-balanced media for a
444 further 10 days, and cell ultrastructure, lipid radiolabeling and lipid content were monitored (Figs. 6
445 and 7). The size of plastoglobules in starved plantlets decreased from *ca.* 0.034 μm^2 cross-sectional
446 area to *ca.* 0.021 μm^2 after 10 days of repletion, which was smaller than that in non-starved plantlets
447 (*ca.* 0.062 μm^2 ; Fig. 6C). Indeed, the size of plastoglobules in control plantlets gradually increased
448 during *in vitro* plant growth, and LDs progressively enlarged in the old control plants (Fig. 6E). This
449 suggests that after 35 days of *in vitro* growth, plantlets suffer stress even on nitrogen-balanced
450 media, which may be due to limited space, gas exchange or light provided by *in vitro* culture
451 (Matuszkiewicz et al., 2019). During the repletion period, the size and number of LDs in parenchyma
452 cells of starved plantlets progressively decreased, and the number of LDs reached a level equivalent
453 to that in control plants (Fig. 6E and F), demonstrating an important remobilization of LDs during the
454 recovery phase. At the same time, the total neutral lipid content in starved plantlets also decreased,
455 from over 16% to less than 7% of total FAs during repletion. The TAGs disappeared completely, while
456 the phytyl-/steryl-ester content remained stable during reversion (Fig. 7A). However, the
457 composition of phytyl-/steryl-esters varied, with a decrease in C16:3 FA and the maintenance of
458 C18:3 (Fig. 7B), suggesting that during repletion, phytyl-esters were remobilized, while SEs were not.
459 Meanwhile, the MGDG content of starved plants gradually increased from 26% to 36%, reaching the
460 MGDG content of the non-starved plants (Fig. 7A).

461 Radiolabeling in TAGs rapidly decreased from 9.0% (± 1.4) to 1.6% (± 0.6) of total labeling during
462 recovery (Fig. 6G), while labeling rapidly increased in PC from 4.4% (± 0.3) to 12.7% (± 0.7) after 3 days
463 of recovery and later in MGDG and DGDG (from 32% and 20% at 7 days of recovery to 39% and 25%
464 after 10 days of recovery, respectively, Fig. 6G), suggesting that FAs stored in TAGs are at least
465 partially mobilized for membrane biogenesis when optimal growth conditions are restored. In
466 contrast, phytyl-/steryl-ester labeling did not significantly decrease during the reversion period,
467 confirming that FAs esterified to sterol moieties were mobilized neither for membrane biogenesis
468 nor for energy production, at least during the first 10 days of the recovery phase.

469

470 **LD/plastid contact sites probably facilitate lipid exchange between plastids and LDs**

471 Our results suggest that FAs derived from MGDG degradation in thylakoids are stored in both
472 plastoglobules and cytosolic LDs. The exchange of molecules between thylakoids and plastoglobules
473 probably takes place via the physical channel between thylakoids and plastoglobules created by the
474 outer leaflet of the thylakoid membrane surrounding the plastoglobules (Austin et al., 2006). MGDG-
475 derived FAs could then transit through this channel to be stored in the plastoglobules. The process by
476 which plastid lipids transit to LDs is less evident. However, using TEM, we frequently observed “close
477 proximity” between plastids and LDs, with either no observable space between the two organelles,
478 or a space smaller than that separating the two membranes of the chloroplast envelope (Fig. 9A). In
479 parenchyma cells of plantlets subjected to nitrogen starvation for 7 days, 62% of LDs, on a total of 85
480 images comprising 302 LDs, were observed in close proximity to at least one chloroplast. In
481 comparison, the frequency of LDs in contact with the plasma membrane was estimated at 18% and
482 with mitochondria at 4%. The size of the zone of proximity between the LD and the plastid averaged
483 416 ± 119 nm (out of 60 measured contact sites), meaning that $20.2 \pm 6.2\%$ of the perimeter of an LD
484 section was engaged in close contact with chloroplasts. In addition, the morphology of some of the
485 proximity zones between LDs and plastids suggest that these are not “accidental” contacts due to the
486 congestion of the cytoplasm by the large vacuole but rather true areas of metabolite transfer, with a
487 change in the curvature of the droplet, which appears to maximize the area of contact with the
488 plastid (see, for example, middle panel in Fig. 9A).

489 To examine the three-dimensional architecture of these putative plastid–LD contact sites, we
490 performed electron tomography. Single-axis tomograms of the contact zone between LDs and the
491 chloroplast were collected from chemically fixed *Arabidopsis* leaves (Fig. 9B–D). Modeling of
492 reconstructed tomograms revealed a narrow apposition of the chloroplast envelope to the LD leaflet
493 over a length of around 400 nm, confirming measurements made on classic electron microscopy
494 images. In small parts of this zone of close proximity, the LD appeared to be in physical contact with

495 the plastid, leaving no apparent gap between the two organelles, while in other parts of the same
496 zone, an intermediate gap of around 4 nm between the LD and the plastid envelope was observed.
497 When close apposition between LD and chloroplast was observed, the outer and inner membranes of
498 the chloroplast envelope appeared closer to each other than anywhere else, as if pressed together,
499 suggesting the presence of a physical factor that favors tight contact between the inner and outer
500 chloroplast membranes on one side and the LD leaflet on the other side.

501

502 **Discussion:**

503 In this study, we demonstrated that galactolipids are genuine lipids of the plastoglobule monolayer.
504 The presence of MGDG with a high ratio of MGDG/DGDG in plastoglobules compared to thylakoids is
505 surprising because, due to its unsaturated acyl chains, the shape of MGDG is that of an inverted
506 cone, which theoretically favors the formation of a negative curvature rather than the positive one
507 needed to form plastoglobules (Graham Shipley et al., 1973). However, as stated by Simidjiev et al.
508 (2000) “lipid–protein interactions play a major part in imposing a (...) configuration in the native
509 membranes.” For example, the CURT1 protein is necessary to induce thylakoid curvature at the grana
510 of *Arabidopsis* chloroplasts (Armbruster et al., 2013), and the addition of the Light-Harvesting
511 Complex II protein to an MGDG mixture dispersed in water modifies the MGDG organization from an
512 inverted hexagonal (HII) phase to a lamellar structure (Simidjiev et al., 2000). Thus, we hypothesized
513 that plastoglobule proteins, such as fibrillins, the main structural proteins found in plastoglobules,
514 favor the formation of plastoglobules. Moreover, the presence and nature of neutral lipids within the
515 plastoglobules may also favor their formation, as described for LDs (Chorlay and Thiam, 2018;
516 Santinho et al., 2021), where the surface tension generated by neutral lipids within the ER membrane
517 favors the budding of LDs. An enrichment in the plastoglobule monolayer of lyso-galactolipids, with
518 their conical shape, could favor the formation of plastoglobules, but only traces of lyso-galactolipids,
519 equivalent to that in thylakoids, were detected in plastoglobules (Fig. 5C). Additional biophysical
520 studies devoted to plastoglobules are undeniably needed to understand the mechanisms of
521 plastoglobule biogenesis.

522 The aim of this study was to decipher the interrelations between the two sites of neutral lipid storage
523 in *Arabidopsis* cells: cytosolic LDs and plastoglobules. We showed that in aging leaves, neutral lipids
524 were stored both in plastoglobules and in cytosolic LDs but that each compartment accumulated
525 different lipids (Figures 3, 4 and 5). Our results demonstrated that, in senescent leaves, the main
526 neutral lipids stored in plastoglobules were, in addition to prenyl quinones, phytol-esters.
527 Plastoglobules are often viewed as containing a substantial amount of TAGs (Kaup et al., 2002;

528 Lippold et al., 2012; Rottet et al., 2015; van Wijk and Kessler, 2017), but the genuine presence of
529 triglycerides in this compartment has been called into question in the review written by Lichtenthaler
530 (2013), who found only a limited amount of TAGs in the plastoglobule fractions obtained from *Ficus*
531 *elastica* and *Tilia* chloroplasts. In this review, Lichtenthaler suggested that the TAGs detected by
532 Tevini and Steinmuller (Steinmüller and Tevini, 1985; Tevini and Steinmüller, 1985) in chloroplasts
533 originated from contamination by cytosolic LDs. Similarly, we found only a limited amount of TAGs
534 (less than 2%mol) in plastoglobules from senescent *Arabidopsis* leaves (Fig. 3), but the FA
535 composition of the plastoglobule TAGs differed from the LD ones, which excludes possible
536 contamination by cytosolic LDs and argues for a genuine presence, although in a scant amount, of
537 TAGs in plastoglobules. However, larger amounts of TAGs are likely stored within plastoglobules from
538 chromoplasts or etioplasts, for example (Dahlin and Ryberg, 1986; Hansmann and Sitte, 1982; Liu et
539 al., 2024; Steinmüller and Tevini, 1985). In agreement with Mueller *et al.* (2015), who described an
540 extra-chloroplastic accumulation of TAGs in *Arabidopsis* leaves in response to heat stress, our results
541 demonstrated that cytosolic LDs were the main compartment for TAG storage in senescent leaves.
542 Given that various abiotic stressors lead to the formation of LDs (Ischebeck et al., 2020; Vries and
543 Ischebeck, 2020, and this study), it is probable that TAGs accumulated in response to these
544 environmental changes are stored within cytosolic LDs and that plastoglobules play only a limited or
545 transient role in this storage. Plastids have the capacity to synthesize TAGs (Kaup et al., 2002; Lippold
546 et al., 2012; Martin and Wilson, 1984), and this synthesis either leads to the formation of transient
547 TAGs that do not accumulate within chloroplasts or occurs under very specific developmental stages
548 (such as in etioplasts or chromoplasts) or environmental stress.

549 It has been proposed that during senescence or different abiotic stress conditions, TAGs are not
550 formed from neo-synthesized FAs but rather represent intermediaries of lipid catabolism, assembled
551 with the FAs released by the degradation of the chloroplast membranes (Cohen et al., 2022;
552 Domínguez and Cejudo, 2021; Kaup et al., 2002; Sakaki et al., 1990). Fan et al. (2017) suggested that
553 upon dark treatment, the accumulating TAG was derived mostly from MGDG and DGDG and that the
554 hydrolyzed MGDG was not converted to DAG before export from the chloroplast, but rather
555 hydrolyzed by lipases to release FFAs that were then exported to the ER. In *Chlamydomonas*
556 *reinhardtii*, Young and Shachar-Hill (2021) showed that in response to nitrogen starvation, FAs
557 derived from polar lipids were incorporated into TAGs and that the FA moieties coming from the
558 TAGs were used to synthesize new membrane lipids during recovery. The lipid composition of LDs
559 and plastoglobules, together with our labeling experiments demonstrated that at least some of the
560 FAs released by the degradation of MGDG during senescence or nitrogen starvation were
561 incorporated into TAGs and phytyl-/steryl-esters. This was confirmed by the analysis of the TAG and

562 phytyl-/steryl-ester composition of *fad2* mutant after nitrogen starvation, which indicates that some
563 C18:3 of chloroplastic origin are incorporated in starvation-induced TAGs and phytyl-/steryl-esters
564 (Fig. 8). Similar results were obtained by Mueller et al. (2017) for heat-induced TAGs. Overall, these
565 results establish that during nitrogen starvation, the C16:3 released by MGDG degradation remains
566 within plastids, mainly esterified as phytyl-esters in plastoglobules, while MGDG-released C18:3 is
567 exported outside plastids into LDs and amasses as TAGs and SEs. This suggests the existence of a
568 segregation process taking place at the plastid envelope during senescence or nitrogen starvation,
569 allowing for the export of C18:3 but not C16:3. To our knowledge, the mechanism responsible for
570 and the reasons for such segregation are unknown. The release of FAs from MGDG may involve
571 lipases, such as Arabidopsis PLASTID LIPASE2 (PLIP2) and HEAT INDUCED LIPASE1 (HIL1) (Higashi et
572 al., 2018; Wang et al., 2018). Under heat stress, HIL1 is responsible for the release of C18:3 at the sn-
573 1 position of MGDG, and less 54:9-TAG (corresponding to 18:3-18:3-18:3 TAG) accumulates in heat-
574 stressed leaves of the *hil1* mutant compared to the wild type. This suggests the incorporation of the
575 C18:3 released by HIL1 from MGDG into TAGs, possibly through the intermediary synthesis of
576 phospholipids (Higashi et al., 2018). Similarly, PLIP2 has also been shown to cleave sn-1 C18:3 from
577 MGDG preferentially (Wang et al., 2018) in response to ABA and possibly to different abiotic
578 stressors. The released C18:3 is then used to produce jasmonate. It would be interesting to
579 determine whether at least one of these galactolipases is also activated during senescence or under
580 nitrogen starvation and could participate in MGDG degradation. In this hypothesis, the C18:3 at the
581 sn-1 position is released, exported outside of the plastid and imported in the ER, probably thanks to
582 FA transporters, such as FATTY ACID EXPORT 1 (FAX1) at the plastid envelope (Li et al., 2015) and
583 AtABCA9 transporter at the ER (Kim et al., 2013), and finally incorporated into TAGs or SEs stored
584 within cytosolic LDs as described for TAGs by Tjellström et al. (2015). The fate of the lyso-MGDG
585 remaining in the plastid is unknown. It could be hydrolyzed by a lipase to provide C16:3 that is
586 further incorporated into phytyl-esters by the PHYTYL-ESTER SYNTHASES PES1 and/or PES2. Another
587 possibility is that lyso-MGDG is directly used as a substrate by PES enzymes, but it remains to be
588 determined whether PES enzymes also accept lyso-MGDG as an acyl donor. Whether other lipases,
589 such as the galactolipid galactosyltransferase SENSITIVE TO FREEZING 2 (SFR2), which catalyzes the
590 formation of DAG and consequently TAG (Moellering et al., 2010), the NPC6 (Nonspecific
591 phospholipase C6) that has been shown to promote galactolipid turnover to TAG production in
592 developing Arabidopsis seeds (Cai et al., 2020), or enzymes with galactosyl hydrolase activity, such
593 as the LD localized CrGH from *C. reinhardtii* (Gu et al., 2021), also participate in the degradation of
594 MGDG and formation of TAGs in response to nitrogen starvation, still needs to be examined.
595 Surprisingly, we also identified SEs in plastoglobules. SEs are generally described as being associated
596 with cytosolic LDs; thus, their presence in plastoglobules suggests contamination of the plastoglobule

597 fractions by LDs. However, the specific composition of the SEs found in plastoglobules, compared to
598 those in LDs (Fig. 4C), excludes any contamination of the plastoglobule fraction by LDs. To exclude
599 any contamination by lipids co-migrating with the SEs in the TLC, we performed LC-MS and
600 unambiguously identified SEs in the plastoglobules (Fig. 4D). SEs have already been described in the
601 elaioplasts of *Brassica napus* anthers (Hernández-Pinzón et al., 1999) and more recently C18:2- and
602 C18:3-sitosterols have also been identified in chromoplast plastoglobules of *Citrus sinensis* (Liu et al.,
603 2024). However, the presence of SEs in plastids is unexplained. Their FA composition, enriched
604 notably in the typically plastidial FAs C16:3 and C18:3, suggests that they are synthesized within the
605 plastids rather than imported. This implies that some sterols are esterified by an unidentified plastid
606 esterase putatively the PES enzymes that were shown to synthesize SEs when expressed in a mutant
607 yeast strain lacking TAG and SE synthesis activity (Lippold et al., 2012). Some sterols have indeed
608 been detected in plastid membranes (Hartmann and Benveniste, 1987) and the import of sterols in
609 elaioplasts has also been suggested by Kobayashi *et al.* (2018). The mechanisms allowing the
610 transport of sterols, from one organelle to another, are still unknown. The import of lipids to plastids
611 occurs mainly through non-vesicular pathways and is believed to occur in part via membrane contact
612 sites (Michaud and Jouhet, 2019). Sterols could also transit from the ER to plastids via contact sites,
613 as ER was observed wrapped around and physically associated with the plastids (reviewed in Bian et
614 al., 2023), or through any other membrane contact site involving a sterol-containing membrane.
615 In *C. reinhardtii*, a physical connection between cytosolic LDs, ER membranes and chloroplasts has
616 been described that likely favors the diffusion of neutral lipids between the ER and plastids through
617 LDs (Goodson et al., 2011; Moriyama et al., 2018). Because we observed frequent apposition of LDs
618 to plastids during nitrogen starvation, we hypothesized that direct transport of lipids from plastids to
619 LDs may occur. Our electron tomography study reinforced this premise by showing that both plastid
620 envelope membranes are closely appressed against each other at the site of LD-plastid contact (Fig.
621 9), which certainly facilitates lipid channeling between plastids and LDs. The fact that the outer and
622 inner envelope membranes are in close proximity at the LD contact site may explain how TGD2
623 (TRIGALACTOSYLTRIACYLGLYCEROL-2), a lipid transporter protein located at the inner membrane of
624 the plastid envelope, can be detected in the LD proteomes of *Arabidopsis* leaves and *C. reinhardtii*
625 (Brocard et al., 2017; Nguyen et al., 2011). We also identified small amounts of galactolipids in LD-
626 enriched fractions, suggesting an export of galactolipids across the plastid envelope to LDs. Such
627 export has already been described during phosphate starvation, where galactolipids replace at least
628 part of the phospholipids in the plastids but also in extraplastidic membranes (Härtel et al., 2000;
629 Klaus et al., 2002) and was suggested to occur through physical contact sites between plastids and
630 mitochondria (Jouhet et al., 2004).

631

632 Further structural studies of LD–plastid contact sites will certainly provide more precision about the
633 configuration and functionality of such structures: do these sites of proximity engage hemifusion
634 events between both organelles, as suggested to occur between plastid and ER (Mehrshahi et al.,
635 2013; Mehrshahi et al., 2014), or rather membrane contact sites? Are some stabilizing or tethering
636 elements involved to maintain these sites of proximity and which ones? Do LD–plastid contacts also
637 occur under other stress conditions? When and how are they established? Could leaf LDs
638 occasionally originate from the plastid envelope, as proposed for *C. reinhardtii* (Fan et al., 2011; Tsai
639 et al., 2015)?

640 The degradation of seed LDs during seed germination has been well described (D’Andrea, 2016;
641 Deruyffelaere et al., 2015; Deruyffelaere et al., 2018; Kelly et al., 2011), but how stress-induced LDs
642 are degraded and contribute to cell homeostasis after returning to normal growth conditions in
643 higher plants remains elusive. Lipids stored in LDs can be degraded by two distinct pathways, namely
644 lipolysis, which relies on the action of lipases, and lipophagy, which refers to the autophagic
645 degradation of LDs in the vacuole (recently reviewed in Bouchnak et al., 2023). It has been
646 demonstrated that both lipolysis and autophagic pathways are involved in TAG remobilization under
647 extended darkness (Fan et al., 2017; Fan et al., 2019). Our study showed that LDs induced by nitrogen
648 starvation disappeared after 10 days of repletion and that TAGs were also fully remobilized, while
649 lipid esters (phytyl- and/or steryl-esters) were not. This suggests the existence of TAG-specific
650 remobilization pathways. Currently, the pathway(s) of neutral lipid remobilization, the fate(s) of TAG-
651 derived FAs and their role in plant recovery are still poorly grasped, and certainly deserve further
652 studies.

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666 **Conflict of Interest**

667 The authors declare that the research was conducted in the absence of any commercial or financial
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674

675 **Author contributions:** DC, JJB and CB designed the research; DB, JJ, and LF performed the lipid LC-
676 MS/MS analyses; DC, HN, and CB performed the experiments and analyzed the data; DC, JJ, JJB, and
677 CB wrote the manuscript.

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FIGURE LEGENDS:

Figure 1: Ultrastructure and lipid composition of Arabidopsis leaves at different ages. **A.** Macroscopic aspect of 6-week-old Arabidopsis plantlets grown at high density; **B.** Ultrastructure of mesophyll cells from 3-week-old (B1), and 6-week-old green (B2), brown (B3) and purple (B4) Arabidopsis leaves. Bar: 5 μm , Chl: chloroplast, St: starch granum, pgl: plastoglobule, LD: lipid droplets; **C.** Quantification of the size and number of plastoglobules and LDs. Values are means \pm SD of the number of measures indicated in brackets **D.** Glycerolipids, phytol- and steryl-esters content in Arabidopsis (Columbia 0) leaves at different ages expressed in μg of fatty acid per mg of fresh weight (FW). Error bars indicate SD of six biological replicates.

Figure 2: Fatty acid distribution of MGDGs, TAGs, phytol- and steryl-esters in 3- to 6-week-old Arabidopsis leaves. The composition of MGDG (A), TAG (B), phytol-esterS (C), and steryl-esters (D) was determined by TLC/GC-FID. The provided values are means and SD of six biological replicates.

Figure 3: Neutral lipid composition of Arabidopsis plastoglobules and lipid droplets from 6-week-old leaves. **A.** Immunoblots of 6-week-old Arabidopsis leaf protein extracts of plastoglobules (PGI), lipid droplets (LD), soluble fraction (Sol.), microsomal fraction (Mic.), total extract (TE), thylakoids (Thyl.) and plastid envelopes (Env.), performed with antibodies raised against plastoglobule (AtFBN1a/AtPGL35), lipid droplet (AtLDAP1), endoplasmic reticulum (Bip), thylakoids (P16) and plastid envelope (Toc75) markers. **B.** Neutral lipid composition (in %mol) of purified plastoglobules determined by TLC coupled to GC-FID (n=7). **C.** Neutral lipid composition (in %mol) of lipid droplets determined by TLC coupled to GC-FID (n=5). **D.** Corrected neutral lipid composition (in %mol) of lipid droplets, after removal of the plastoglobule contaminating lipids. The provided values are means and SD of different biological replicates.

Figure 4: Fatty acid distribution of neutral lipids in plastoglobules and lipid droplets from 6-week-old Arabidopsis leaves. The composition of TAGs (A), phytol-esters (B) and steryl-esters (C) was determined by GC-FID. The identity of the steryl-esters was also determined by LC-MS/MS (D). The global FA composition (E) of plastoglobules and lipid droplets was calculated based on GC-FID quantifications. Values are means \pm SD of 7 (plastoglobules) and 5 (LDs) biological replicates.

Figure 5: Molecular species distribution of DGDG (A) and MGDG (B) from lipid droplets, plastoglobules and thylakoids of 6-week-old Arabidopsis leaves. The composition was determined by LC-MS/MS. Means and SD of 5 (LDs), 7 (plastoglobules) and 10 (thylakoids) biological replicates are shown. ** and *** represent statistically significant differences at p-value ≤ 0.01 and p-value ≤ 0.005 , respectively, as determined by Wilcoxon test. C: Repartition of the galactolipids within LD, plastoglobule and thylakoid fractions.

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Figure 7: Glycerolipid analyses of Arabidopsis plantlets during nitrogen starvation and reversion. A. Glycerolipid distribution (in % of lipids); B. Neutral lipid fatty acid content (in $\mu\text{g}/\text{mg}$ fresh weight). Two-week-old Arabidopsis plantlets were grown for 10 days on nitrogen balanced medium (N^+) or on nitrogen deprived medium (N^- , grey area) and their lipid composition determined by TLC/GC-FID at day 0 (D0), 3 (D3), 7 (D7) and 10 (D10). Plantlets were then transferred to nitrogen balanced medium for reversion, and their lipid composition similarly monitored after 5 days (D15) and 10 days (D20). Green bars: plantlets grown on nitrogen balanced medium; orange bars: plantlets submitted to nitrogen starvation for 10 days. For clarity reason, values of D0 are presented twice: with control (green) as well as with starved (orange) samples. Values are means \pm SD of 4 biological replicates. P-/S-Esters : Phytyl-/Steryl-esters.

Figure 8: Fatty acid composition of neutral lipids from Col0 and *fad2* Arabidopsis plantlets during nitrogen starvation. Two-week-old Arabidopsis plantlets were grown for 10 days on nitrogen

deprived medium and lipid extracted. The fatty acid compositions of phytyl-/steryl-esters (A) and TAGs (B) were determined by TLC/GC-FID before starvation (D0), and after 10 days of starvation (D10 N-). Green bars: *Col0* wild type; blue bars: *fad2* mutant. Values are means \pm SD of 4 or 6 biological replicates for *Col0* or *fad2* respectively.

Figure 9: Morphology of the plastid-lipid droplet contact monitored by tomography. The ultrastructure was determined on chemically fixed Arabidopsis leaves subjected for 7 days to nitrogen starvation. **A.** Electron micrographs showing close proximity (white arrow heads) between LD and chloroplast. Bar: 0.5 μ m. **B.** Overview of the contact between a chloroplast and a LD. Bar: 0.5 μ m. **C.** Tomographic slices of plastid-LD contact site shown in B., highlighting the appression of both plastid envelope membranes with the LD monolayer (white arrow heads). Bar: 0.1 μ m. LD: lipid droplet; Env.: plastid envelope; iM: inner membrane of the plastid envelope; oM: outer membrane of the plastid envelope; PG: plastoglobule; Thyl.: thylakoids. The thickness of one tomographic slice is estimated to be around 0.23 nm. **D.** 3D segmentation of C; yellow: LD surface, light pink: outer envelope membrane, purple: inner envelope membrane, green: thylakoids, cream: starch granule.

Figure S1: Fatty acid distribution of phosphatidylcholine (A), phosphatidylinositol (B), phosphatic acid (C), a mix of phosphatidylethanolamine and phosphatidylglycerol (D), and digalactosyldiacylglycerol (E) in leaves from 3- to 6-week-old Arabidopsis plantlets. The fatty acid distribution was determined by TLC/GC-FID. Values are means \pm SD of 6 biological replicates.

Figure S2 : LC-MS and GC-FID responses for Steryl Ester analysis

(A) LC-MS analysis of cholesteryl esters. A solution of cholesteryl ester standards, containing equal quantities (in mass) of 18:0-Cholesterol (C79409, Sigma Aldrich, \geq 96%), 18:1-Cholesterol (C9253, Sigma Aldrich, \geq 98%), and 18:2-Cholesterol (C0289, Sigma Aldrich, \geq 98%) was prepared and a volume corresponding to 1.25 pg of each standard was injected and analyzed by LC-MS (MRM). A difference in ionisation yield is observed between the difference SEs, with 18:0-Cholesterol peak area: 2.464e6, 18:1-Cholesterol peak area:1.465e7, and 18:2-Cholesterol peak area:2.95e7 (arbitrary unit).

(B). **GC-FID analysis of the same standard solution.** An aliquot of the cholesteryl ester solution was first transesterified in H₂SO₄ (2,5% v/v) in methanol, containing 5 mg/mL C17:0 as internal standard. After 1h at 85°C, FAMES were extracted using 500µL Hexane and 1 mL H₂O. 1 µL of the hexane phase was injected on GC-FID. Ionisation yield is similar for the different FAMES, with 18:0 peak area: 1605, 18:1 peak area: 2007, and 18:2 peak area: 2136 (arbitrary unit).

Figure S3: Species distribution of polar lipids in plastoglobules and lipid droplets purified from 6-week-old Arabidopsis leaves, determined by LC-MS. Lipids from plastoglobule, lipid droplet and thylakoid fractions have been extracted and the distribution of species in SQDG (A), phosphatidylglycerol (B), phosphatidylinositol (C) and phosphatidylcholine (D) determined by LC-MS/MS. Means of 3 replicates for lipid droplets and plastoglobules and 5 replicates for thylakoids are shown.

Figure S4: Fatty acid distribution of glycerolipids from Arabidopsis plantlets, during nitrogen starvation and reversion. Two-week-old Arabidopsis plantlets were transferred for 10 days on nitrogen balanced medium or on nitrogen deprived medium (grey area) and their lipid composition determined by TLC/GC-FID at day 0 (D0), 3 (D3), 7 (D7) and 10 (D10). Plantlets were then transferred back to nitrogen balanced medium for reversion, and their lipid composition similarly determined after 5 days (D15) and 10 days (D20). Green bars: plantlets grown on nitrogen balanced medium; orange bars: plantlets submitted to nitrogen starvation for 10 days. For clarity, values of D0 are presented twice: with control (green) as well as with starved (orange) samples. Values represent percents of total FAs, and are means ± SD of 4 biological replicates. FFA: Free Fatty acid, P-/S-Esters : Phetyl-/Steryl-esters.

Figure S5 : Fatty acid composition of PC and MGDG from 2 week old Arabidopsis plantlets. The fatty acid composition of PC (A) and MGDG (B) from Col0 wild type Arabidopsis and *fad2* mutant was determined by TLC/GC-FID after 2 weeks *in vitro* growth on nitrogen-balanced medium. Green bars: wild type Col0; blue bars: *fad2* mutant. Values are means ± SD of 6 biological replicates.

Table S1: Distribution of ¹⁴C originating from [¹⁴C]acetate among lipids extracted from aerial parts of Arabidopsis plantlets. Two-week-old plantlets were incubated for 2 h with [¹⁴C]acetate (time 0), rinsed, and grown *in vitro* on N-balanced (Control plants) or N-deprived (N-starved plants) medium

for 10 days and then transferred on N-balanced medium for recovery during 10 additional days. Samples were harvested at different times during the starvation period (3, 6 and 10 days), and the recovery period (13, 15, 17 and 20 days). All data points are the means \pm SD of five biological replicates.

Method S1: LC-MS/MS analyses

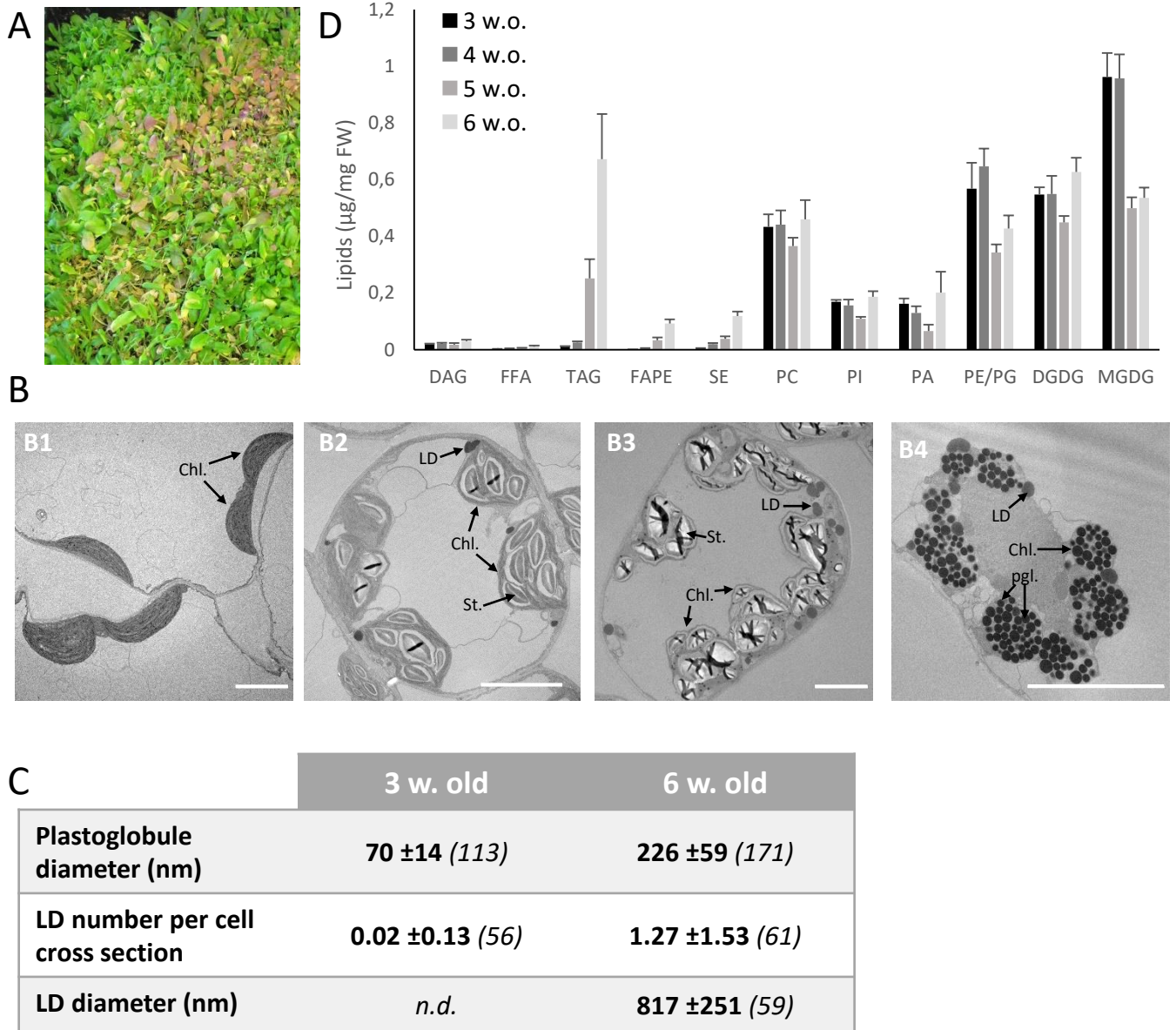
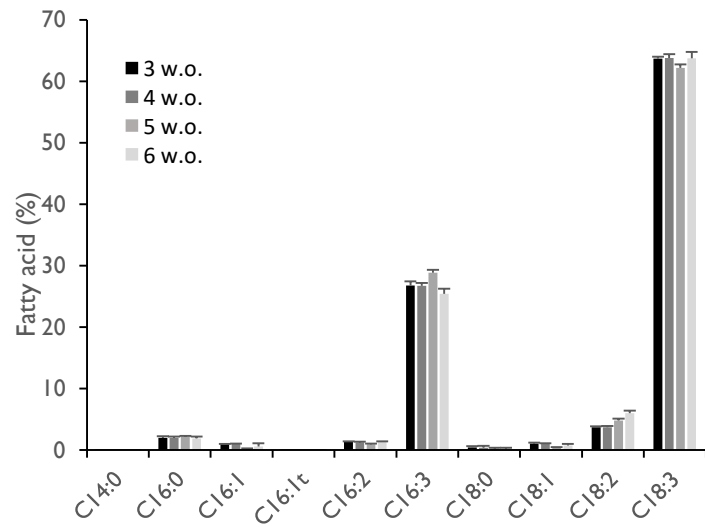
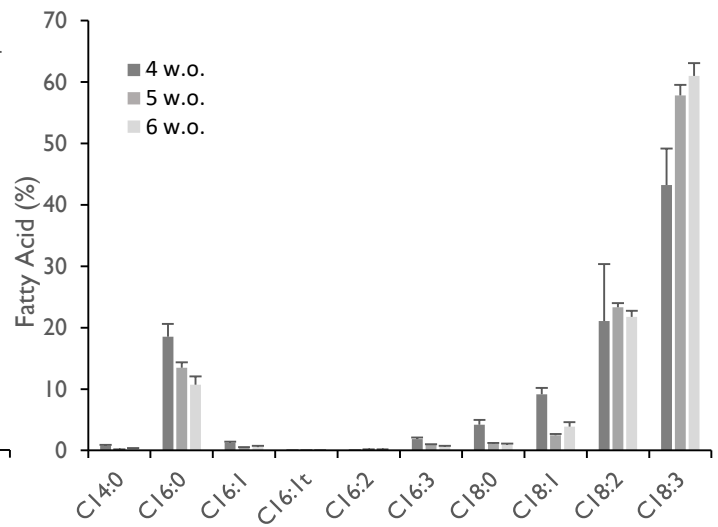


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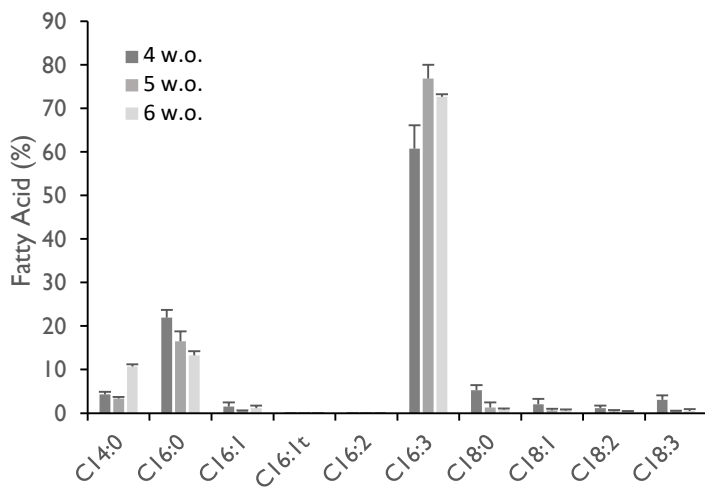
A. MGDG



B. TAG



C. Phytyl-esters



D. SEs

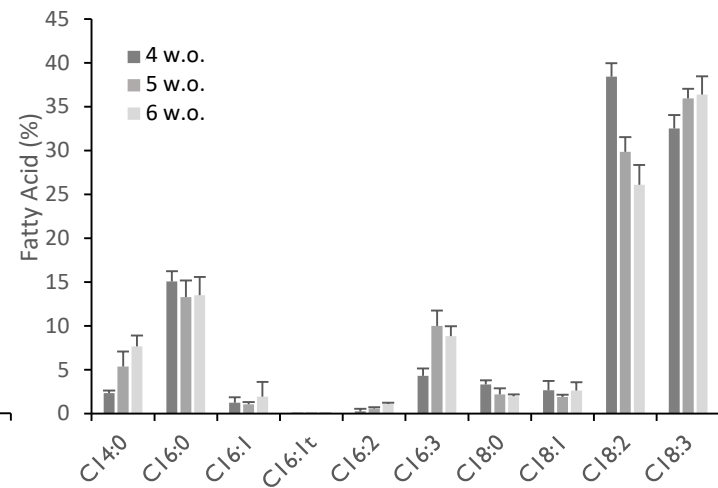


Figure 2: Fatty acid distribution of MGDGs, TAGs, phytyl- and steryl-esters in 3- to 6-week-old *Arabidopsis* leaves. The composition of each lipid class was determined by TLC/GC-FID. The provided values are means and SD of six biological replicates.

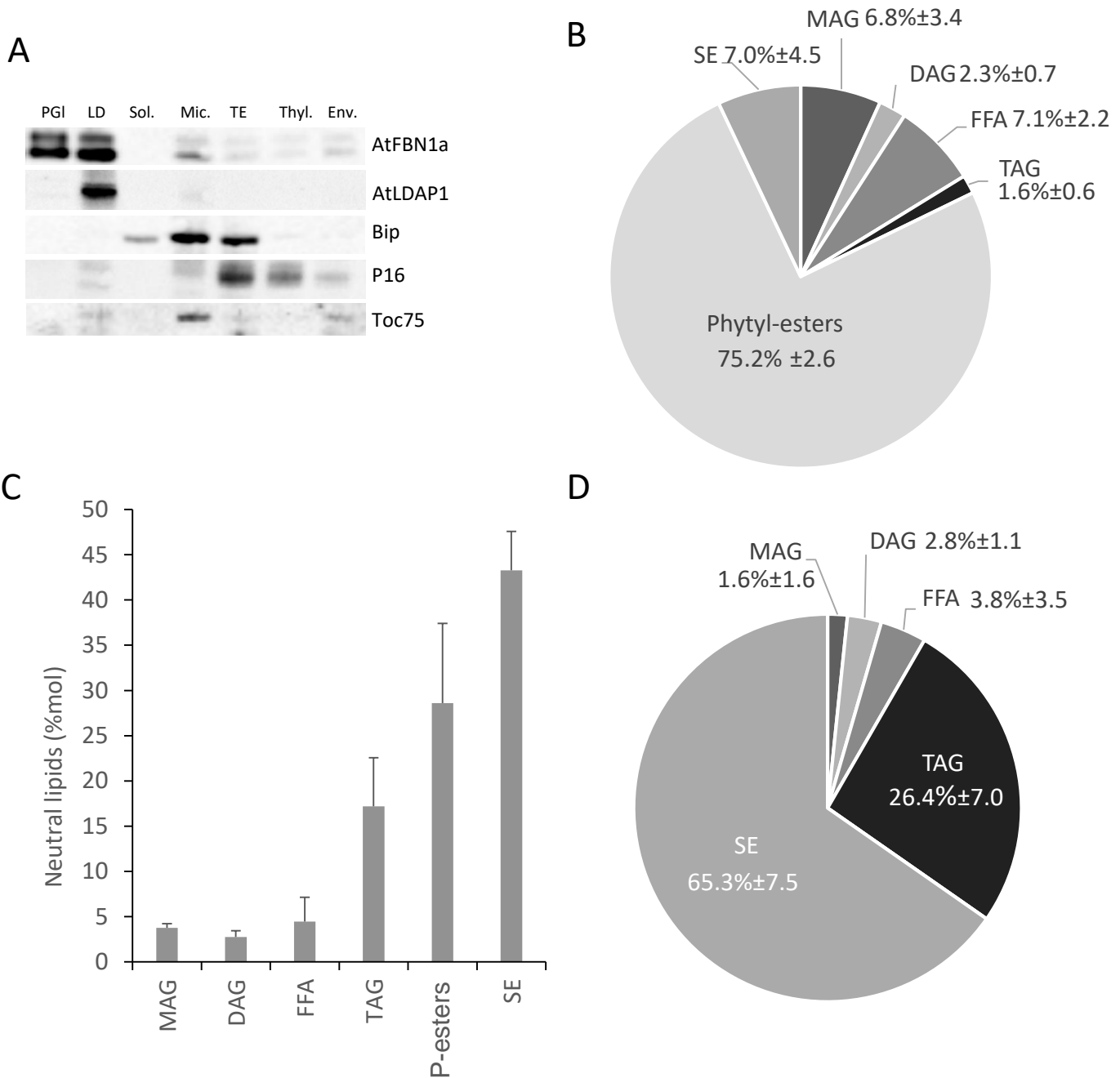


Fig.3: Neutral lipid composition of Arabidopsis plastoglobules and lipid droplets from 6-week-old leaves. A. Immunoblots of 6-week-old Arabidopsis leaf protein extracts of plastoglobules (PGI), lipid droplets (LD), soluble fraction (Sol.), microsomal fraction (Mic.), total extract (TE), thylakoids (Thyl.) and plastid envelopes (Env.), performed with antibodies raised against plastoglobule (AtFBN1a/AtPGL35), lipid droplet (AtLDAP1), endoplasmic reticulum (Bip), thylakoids (P16) and plastid envelope (Toc75) markers. B. Neutral lipid composition (in %mol) of purified plastoglobules determined by TLC coupled to GC-FID (n=7). C. Neutral lipid composition (in %mol) of lipid droplets determined by TLC coupled to GC-FID (n=5). D. Corrected neutral lipid composition (in %mol) of lipid droplets, after removal of the plastoglobule contaminating lipids. The provided values are means and SD of biological replicates. P-esters: Phytol-esters.

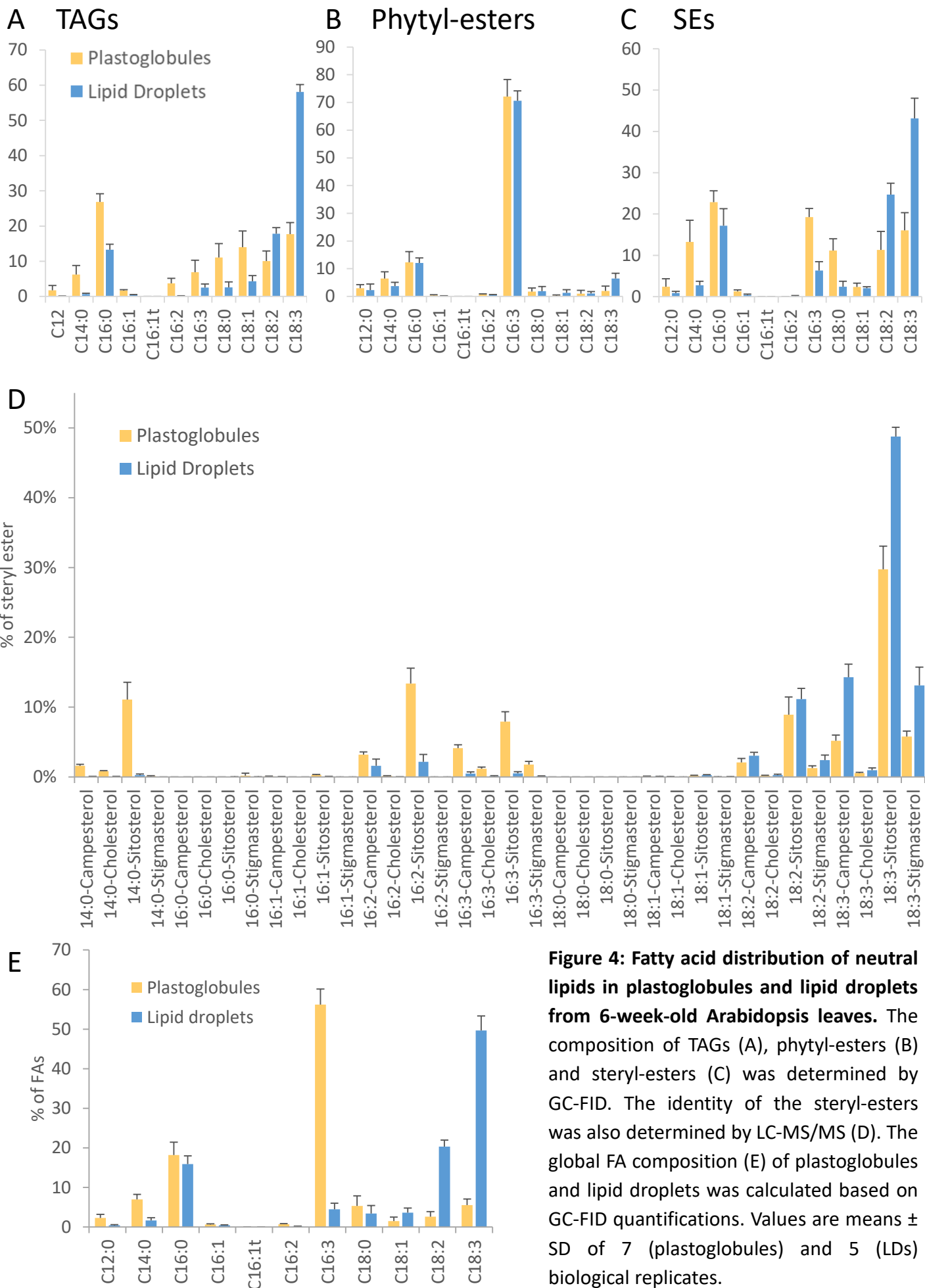
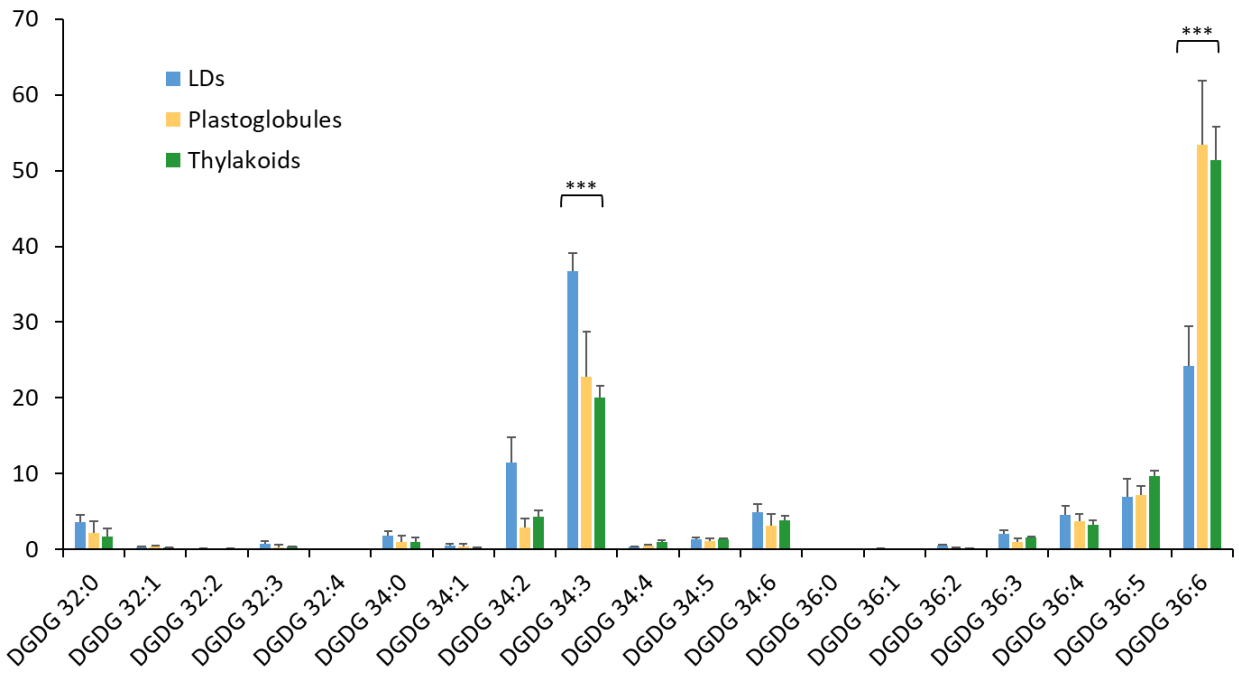
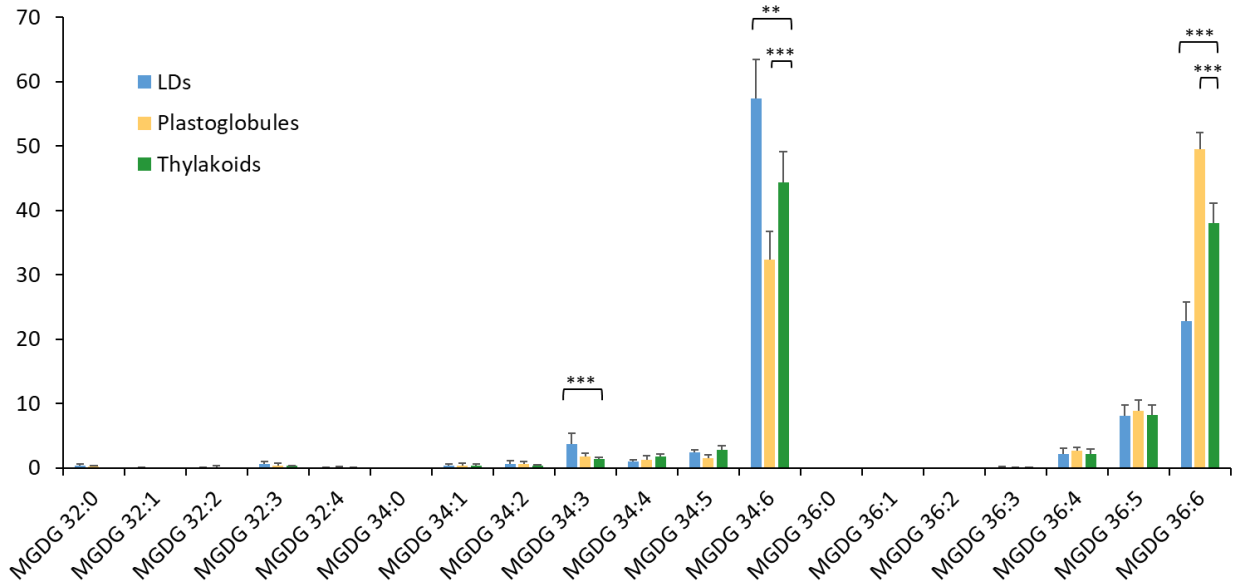


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A DGDG



B MGDG



C Galactolipids

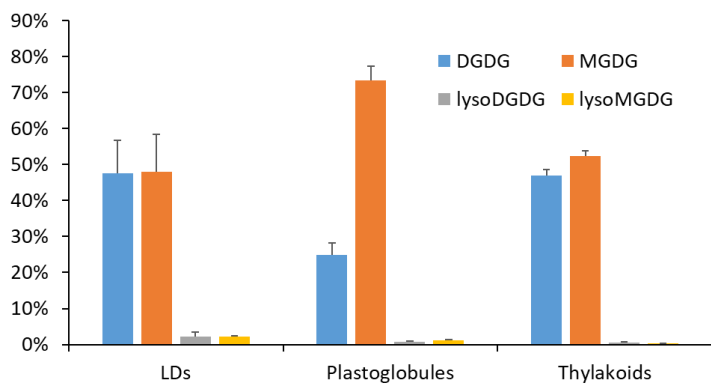


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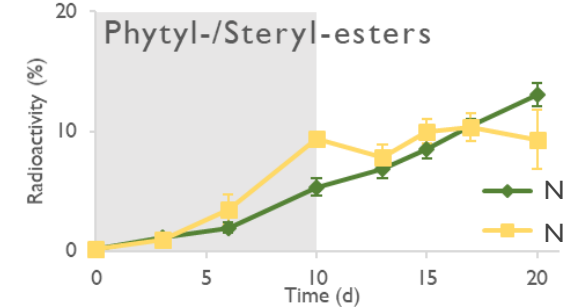
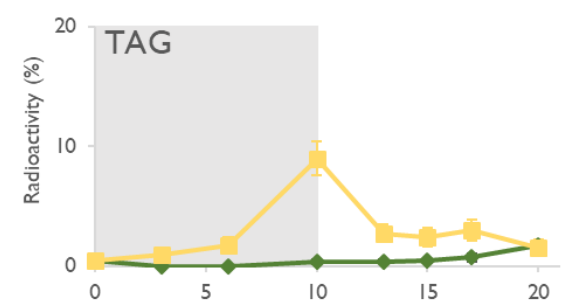
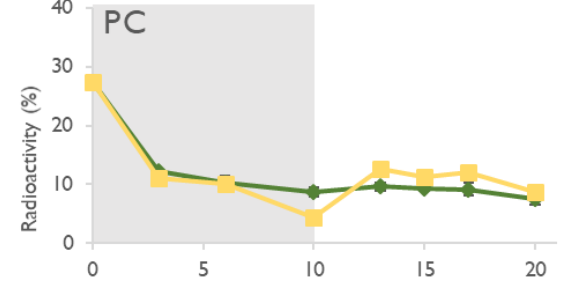
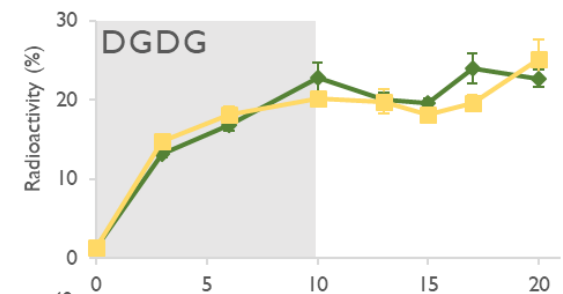
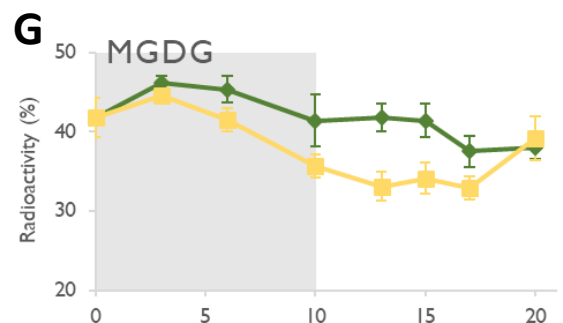
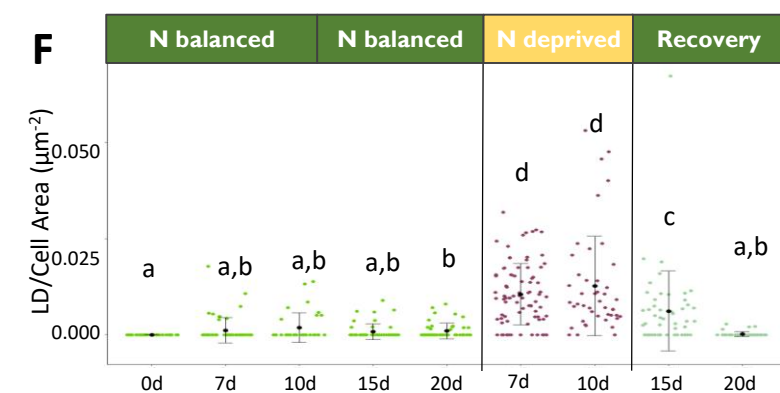
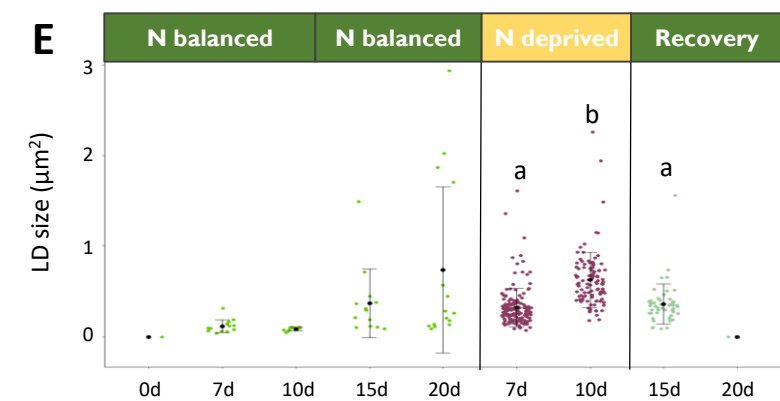
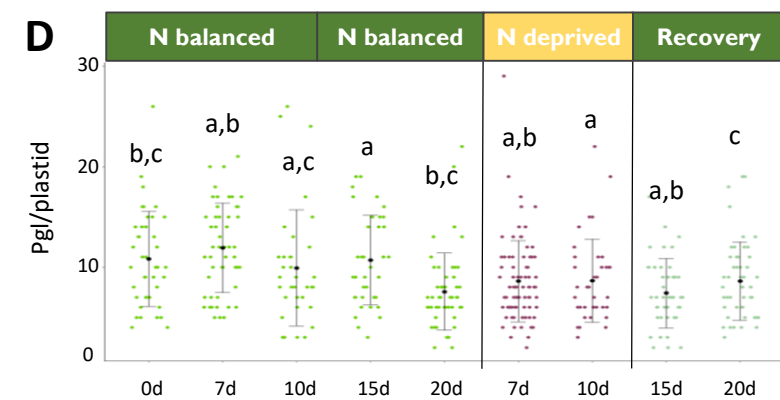
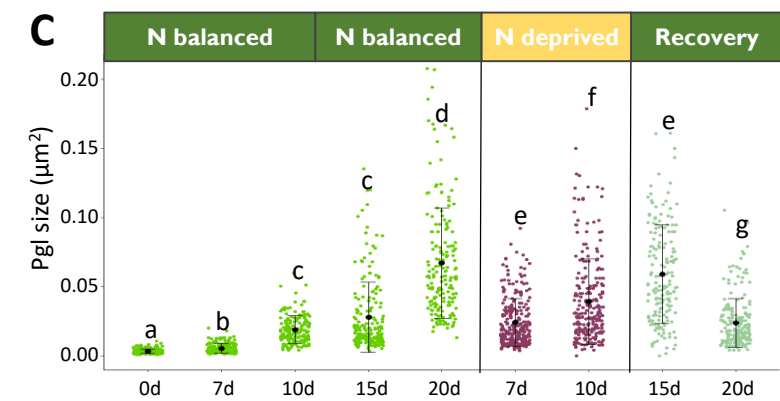
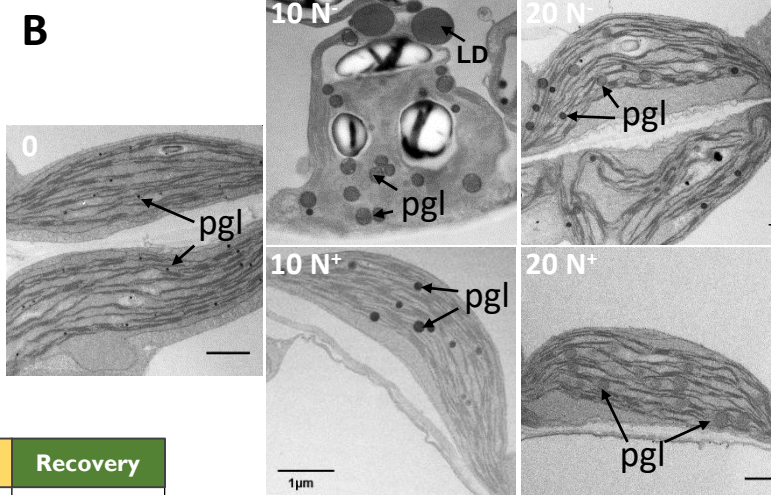
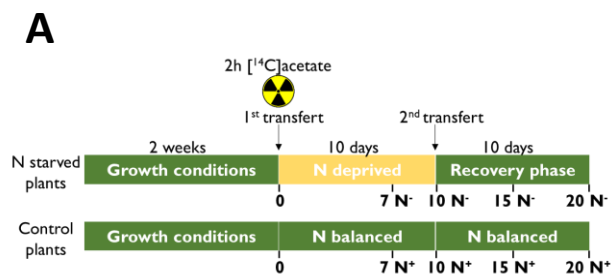


Fig. 6: Effects of nitrogen starvation followed by a repletion period on Arabidopsis leaf cell ultrastructure and lipid metabolism. **A.** Scheme of the experimental procedure showing the labelling pulse of two-week-old plantlets, the 10 days of starvation on nitrogen depleted medium and the recovery phase for 10 supplemental days on nitrogen balanced medium. The control plantlets followed the same experimental procedure excepted that they were always transferred to nitrogen balanced medium. **B.** Ultrastructure of chemically fixed Arabidopsis leaves after 10 days of nitrogen starvation ($10N^-$) or 10 days of controlled growing conditions ($10N^+$) and 10 supplemental days of repletion ($20N^-$ or $20N^+$), observed by transmission electron microscopy. Scale bar: 1 μm ; Quantification of the size of plastoglobules (**C.**, cross-section area, in μm^2), number of plastoglobules per plastid (**D.**), size of LDs (**E.**, cross-section area, in μm^2), and number of LDs per cell section area (**F.**, μm^{-2}) in response to nitrogen starvation and after repletion. Bar = 1 μm . Pgl: plastoglobules. (more than 40 quantifications per parameter were obtained on at least 2 independent plants). **G.** Kinetics of the radiolabelling of MGDG, DGDG, phytyl-/steryl-esters and TAGs during nitrogen starvation and recovery phases. Grey area corresponds to nitrogen starvation period. Values are means \pm SD of five biological replicates.

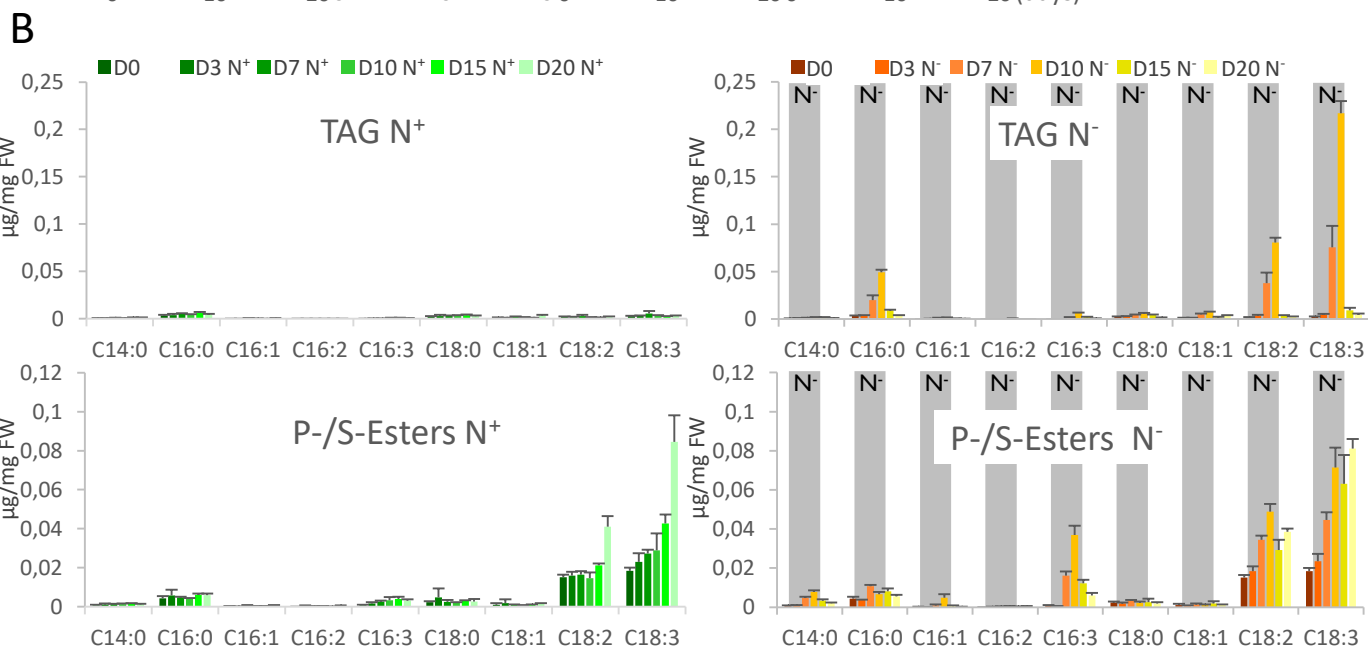
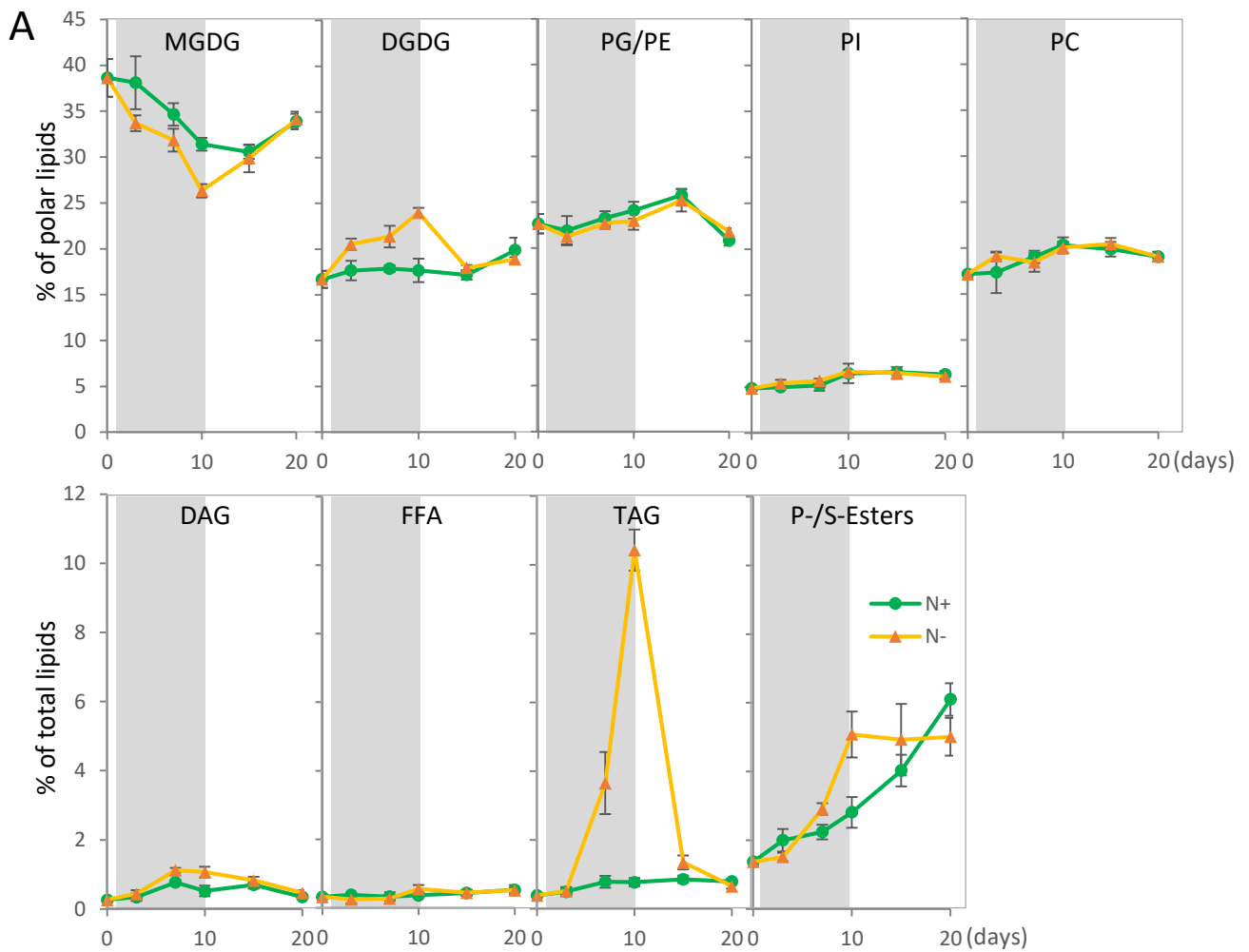


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A. Phytyl-/Steryl-esters

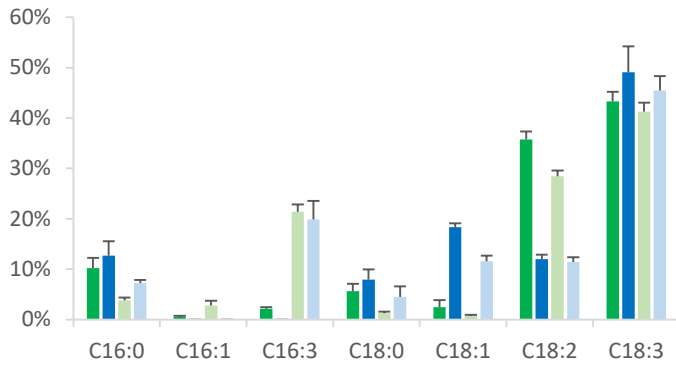
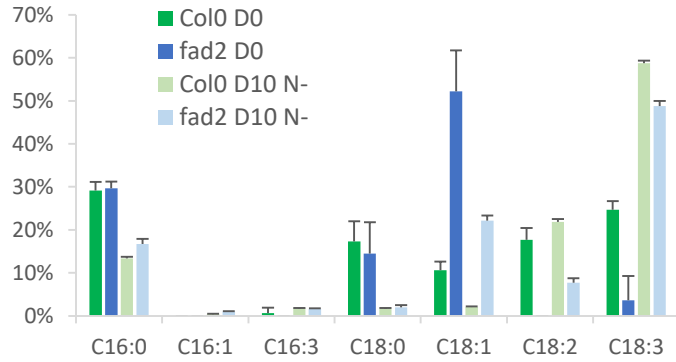
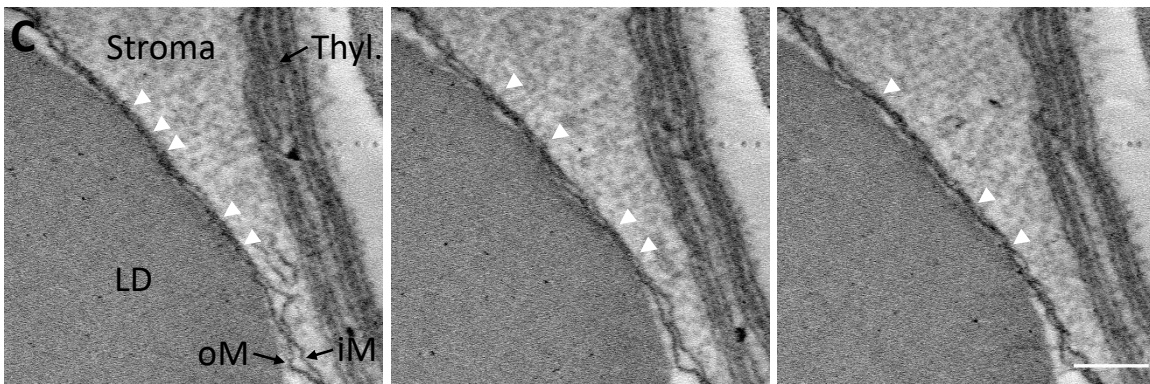
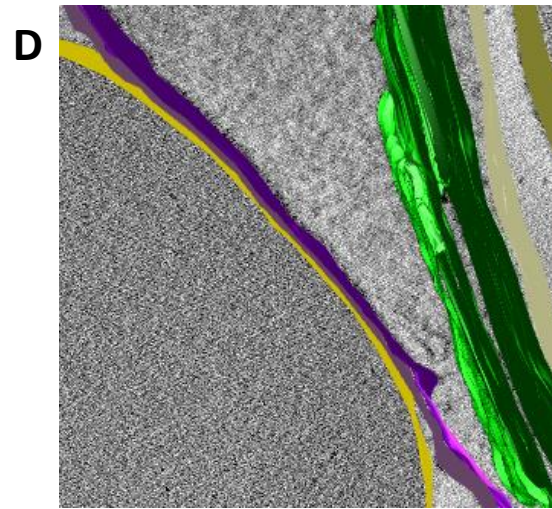
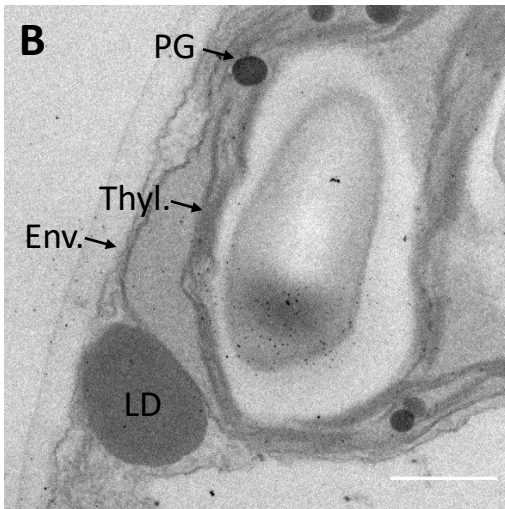
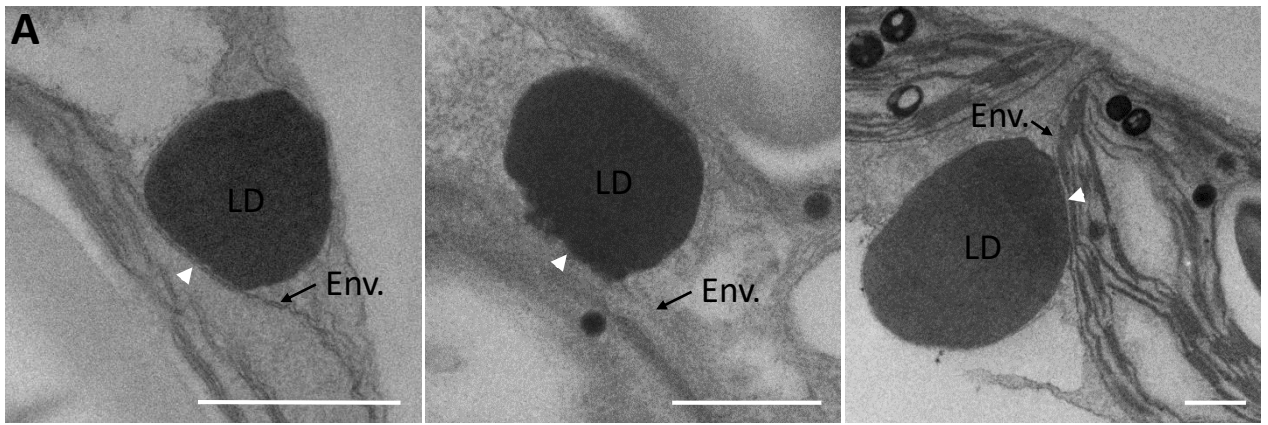


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B. TAGs





Z = -25

Z = 0

Z = +25

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