TITLE:

DIFFERENTIAL EFFECTS OF REACTIVE OXYGEN SPECIES ON AUTOPHAGY IN ARABIDOPSIS

RUNNING TITLE:

Redox regulation of autophagy

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KEYWORDS

Autophagy, stress, reactive oxygen species, singlet oxygen, superoxide radical, hydrogen peroxide, redox state, Arabidopsis thaliana.

HIGHLIGHT

This research show that the differential effects of ROS on autophagic flux correlates with their effects on cytoplasmic redox state.

ABSTRACT

Autophagy plays a key role in the responses to different stress condition in plants, providing both pro-life and pro-death functions. Reactive oxygen species (ROS), are common modulators of stress responses, having both toxic and signaling functions. In this context, the relationship between ROS and autophagy regulation remains unclear, and in some aspect contradictory. In this study, we employed pharmacological and genetic approaches to investigate the effects of radical superoxide (O_2^-), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) on the cytoplastic redox state and autophagic flux in Arabidopsis thaliana. Ours results demonstrated that oxidative treatments with H2O2 and MV, which drastically increased the oxidized state of the cytoplasm, reduced the autophagic flux. Conversely, singlet oxygen, which did not have significant effects on the cytoplasmic redox state, increased the autophagic flux. Additionally, our findings indicated that after H2O2 and high light treatments and during the recovery period, the cytoplasm returned to its reduced state, while autophagy was markedly induced. In summary, our study unveils the differential effects of ROS on the autophagic flux, establishing a correlation with the redox state of the cytoplasm. Moreover, it emphasizes the dynamic nature of autophagy in response to oxidative stress and the subsequent recovery period.

KEYWORDS

Autophagy, stress, reactive oxygen species, singlet oxygen, superoxide radical, hydrogen peroxide, redox state, Arabidopsis thaliana.

ABBREVIATIONS

ATG: autophagy genes
Conc A: Concanamicina A
GFP: green fluorescent protein
GSH: reduced glutathione
GSSG: oxidized glutathione
MV: methyl viologen
roGFP: redox-sensitive green fluorescent proteins
ROS: reactive oxygen species

97 **INTRODUCTION**

98 Plants as sessile organisms are constantly exposed to different biotic and abiotic stress
99 conditions, so they have evolved integrated defense and acclimation mechanisms to cope with
100 adverse conditions. A common effect induced in plants by different stress conditions is the
101 increase in the generation of reactive oxygen species (ROS), such as superoxide (O_2^-), hydrogen
102 peroxide (H_2O_2), singlet oxygen ($^1O_2$), and the hydroxyl radical (-OH), at different subcellular
103 compartments, such as chloroplasts, mitochondria, peroxisomes, cytosol and the apoplast (Apel
104 and Hirt, 2004). ROS are potent oxidants, that depending on their intensity, duration, and
105 location site of production within the cell can modify different biomolecules, affecting the redox
106 state in different subcellular compartments, provoking oxidative stress, inducing senescence
107 symptoms and cell death (Apel and Hirt, 2004; Trippi *et al.*, 2009; Muñoz *et al.*, 2012; Robert *et*
108 *al.*, 2018). Furthermore, due to their high reactivity and toxicity, the maintenance of tightly
109 regulated ROS levels becomes crucial for plant acclimation, adaptation, and survival, facilitated
110 by the activity of a sophisticated plant antioxidative system. In this way, controlling the different
111 types of ROS produced, their intensity, duration, and site of production has different
112 implications in modulating growth, development, and stress responses (Melchiorre *et al.*, 2009;
113 Miller *et al.*, 2009; Robert *et al.*, 2009). Consequently, ROS are recognized as critical signaling
114 molecules, and the cellular redox state is considered an intensive property of the living system
115 that integrates information from metabolic and environmental cues controlling plant growth
116 and acclimation responses, as well as cell suicide events (Breusegem et al., 2001; Vranova et al.,
117 2002; Breusegem & Dat, 2006; Miller et al., 2008; Baxter *et al.*, 2014; Choudhury *et al.*, 2017;
118 Fichman and Mittler, 2020).

119 In plants, chloroplast, mitochondria and peroxisome, which are redox active organelles
120 where carbon and energetic metabolisms take place, are major sources of ROS production (Apel
121 and Hirt, 2004). In chloroplasts, the photosynthetic process involves physical, biophysical, and
122 biochemical redox reactions with different reaction rates. In consequence, oxygen became a
123 potent univalent electron or energy acceptor from highly activated intermediates under
124 conditions of excess excitation energy (EEE) (Karpinski *et al.*, 1999). ROS produced in the
125 chloroplasts are particularly important in the retrograde signaling of EEE conditions, affecting
126 nuclear gene expression, acclimation responses as well as programmed cell death (Karpinski *et*
127 *al.*, 1999; Breusegem *et al.*, 2001; Breusegem and Dat, 2006; Bechtold *et al.*, 2008; Fichman and
128 Mittler, 2020).

129 Likewise, several stress conditions in plants have been also shown to be capable of
130 triggering or increasing autophagy (Signorelli *et al.*, 2019; Wang *et al.*, 2021). Autophagy is an
131 ancient and conserved process in eukaryotes by which different cellular components are
132 delivered to vacuole/lysosome to be degraded, and is essential for plant energetic metabolisms,
133 growth, development and response to different stress conditions (Su *et al.*, 2020; Wang *et al.*,
134 2021). Different types of autophagy have been identified in plants (Yoshimoto, 2012).
135 Macroautophagy (hereafter termed autophagy) is the type of autophagy most thoroughly
136 studied in all organisms. The autophagic process is initiated, and mainly modulated in the
137 cytosol, by the formation of a double membrane cup, the phagophore, that expands to engulf
138 macromolecules and cellular components to finally form a double-membrane vesicle called
139 autophagosomes. The autophagosomes are then transported and fused with the vacuole,
140 facilitating the delivery of their cargo for subsequent degradation (Yoshimoto, 2012; Yoshimoto
141 and Ohsumi, 2018). The several steps of the autophagic process are coordinated by ATG

142 (AuTophaGy) proteins as well as by some components of the endosome pathway, as recently
143 reported (Zhuang *et al.*, 2015; Yoshimoto and Ohsumi, 2018; Robert *et al.*, 2021). While early
144 evidence pointed to a strong transcriptionally regulation of autophagy in response to nutritional
145 starvation conditions and foliar senescence (Yoshimoto *et al.*, 2004; Breeze *et al.*, 2011), recent
146 studies have shed light on the widespread modulation of autophagy initiation and progression
147 by post-translational modifications of core autophagy proteins. These modifications primarily
148 occur at the cytoplasmic level in response to environmental cues (Feng *et al.*, 2015; Yang *et al.*,
149 2019; Qi *et al.*, 2021).

150 It has been suggested a positive correlation between ROS generation and the autophagic
151 process, where ROS can serve as both sources of damage and signals (Xiong *et al.*, 2007*a*,*b*;
152 Signorelli *et al.*, 2019). Autophagy promotes the degradation of ROS-overproducing damaged
153 organelles including chloroplasts, mitochondria, and peroxisomes, thus fulfilling antioxidant
154 roles (Xiong *et al.*, 2007*b*; Shibata *et al.*, 2013; Nakamura *et al.*, 2018; Oikawa *et al.*, 2022).
155 Supporting this notion, observations in different autophagy-mutant plants demonstrate the
156 accumulation of oxidized and ubiquitinated proteins (Xiong *et al.*, 2007*a*; Munch *et al.*, 2014),
157 high levels of H_2O_2 (Yoshimoto *et al.*, 2009), increased amounts of total glutathione (Masclaux-
158 Daubresse *et al.*, 2014), accelerated senescence (Doelling *et al.*, 2002; Hanaoka *et al.*, 2002;
159 Yoshimoto *et al.*, 2004), and hypersensitivity to different stress conditions including oxidative
160 stresses (Xiong *et al.*, 2007*b*; Liu *et al.*, 2009; Zhou *et al.*, 2013), indicating a significant redox
161 imbalance. On the other hand, oxidative treatments with H_2O_2 and methyl viologen (MV) have
162 been shown to induce autophagy (Xiong *et al.*, 2007*b*; Pérez-Pérez *et al.*, 2012*b*). Likewise,
163 autophagosome formation triggered by nutritional starvation and saline stress in Arabidopsis
164 roots depends on the NADPH-oxidase complex, a major source of ROS in the apoplast, which is
165 essential for local and systemic redox signal transduction (Liu *et al.*, 2009). However, the
166 induction of autophagy under osmotic stress was shown to be NADPH oxidase-independent,
167 suggesting that different signaling pathways regulate autophagy induction, and in certain
168 scenarios are dependent on ROS signaling (Liu *et al.*, 2009). More recently, it has been
169 hypothesized that H_2O_2 accumulation and membrane damage may mediate chlorophagy
170 induced by UV and high light treatments in Arabidopsis leaves (Izumi *et al.*, 2017; Woodson,
171 2019). Consequently, it is plausible that autophagy induction occurs as a response to the
172 accumulation of cellular components that have been damaged. Notably, recent studies
173 conducted in yeast, animals and Chlamydomonas have revealed that certain proteins involved
174 in the autophagic process could be inhibited by ROS in *in vitro* experiments or artificial oxidative
175 stress *in vivo*, indicating that ROS may inhibit the function of some autophagy-related proteins
176 (Pérez-Pérez *et al.*, 2014, 2016; Frudd *et al.*, 2018). These findings raise apparently opposite
177 evidence regarding the redox regulation of autophagy. Hence, the picture about the relationship
178 among different ROS, subcellular redox changes, and autophagy regulation in plants is still
179 unclear. Under this scenario, our study aimed to address the effects of different ROS on
180 autophagy in plants.

181 Herein, we provide evidence that oxidative treatments with H_2O_2 and MV, which
182 drastically increased the oxidized state of the cytoplasm, led to a reduction in the autophagic
183 flux in Arabidopsis seedlings. Likewise, after the oxidative treatments and during the subsequent
184 recovery period, the cytoplasm returned to its reduced state while autophagy was markedly
185 induced. On the other hand, pharmacological and genetic approaches that enhance the
186 generation of $^1O_2$ resulted in an increased autophagic flux in Arabidopsis seedlings, but no

187 significant effects were observed on the cytoplasmic redox state. In summary, our findings
188 unveil the distinct effects of ROS on the autophagic flux, highlighting a strong correlation
189 between the oxidized state of the cytoplasm and the inhibition of autophagy.
190
191 **MATERIALS AND METODS**
192 **Plant materials and growth conditions.**
193 *Arabidopsis thaliana* ecotype Columbia (Col-0) was used in this study. 35S:GFP-ATG8a transgenic
194 seeds (CS39996) (Thompson et al., 2005) were obtained from the Arabidopsis Biological
195 Resource Center. The cytosol-targeted fluorescent redox sensors GFP2-Orp1 or GRX-roGFP2
196 were provided by Dr. Markus Schwarzländer. Fluorescent (*flu*) and ferrochelatase 2 (*fc2*)
197 mutants were provided by Dr. Jesse Woodson. We introgressed the GFP-ATG8a, GFP2-Orp1 and
198 GRX-roGFP2 transgenes into the *flu* and *fc2* mutant lines by genetic crosses. Homozygous
199 offspring were then selected from the F3 backcross generations.
200 Seeds were surface-sterilized and sown on culture plates containing nutrient-rich Murashige and
201 Skoog (MS) solid medium lacking sucrose and containing 1 % agar. After sowing, plates were
202 kept in darkness at 4°C for 2 to 3 days, and then transferred to a growth chamber under a 16 h
203 light/8 h dark photoperiod. Photosynthetic photon flux density was 110 µmol m^{-2} sec^{-1}. Average
204 temperature was 22°C. Plants were grown for 7 days under these conditions and then passed to
205 MS liquid medium to carry out the different treatments. For salt and osmotic stresses, NaCl (50
206 mM) and mannitol (100 mM) were used, respectively. Oxidative treatments were performed by
207 the supplementation of the MS liquid medium with 1 µM methyl viologen (MV), 10 mM H_2O_2,
208 20 µM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), 20 µM methylene blue (MB). To
209 scavenge superoxide and peroxide, 10 mM tiron and 10 mM benzoic acid (BA) were co-
210 administrated, respectively. 10 mM dithiothreitol (DTT) was used as a reducing agent for protein
211 disulfide bonds.
212
213 **Confocal fluorescence microscopy and image analysis.**
214 Confocal microscopy was performed on an Inverted microscope Nikon Eclipse CZ1. 35S:GFP-
215 ATG8a expressing plants were observed with a 488-nm argon laser (BP 495–530 nm). To analyze
216 autophagic bodies accumulation, 1 µM Concanamycin A was added to the different treatments.
217 The number of autophagic bodies in epidermal cells was quantified after 16 h of treatments
218 using ImageJ by counting their numbers in the central vacuole of multiple cells in sections of
219 2500 μm^2. Cytosol-targeted fluorescent redox sensors GFP2-Orp1 or GRX-roGFP2 were excited
220 at 488 nm and 405 nm and fluorescent emissions were collected at 505-535 nm. Subsequently,
221 a single ratiometric image was generated using the ImageJ software, where the value of each
222 pixel is the product of the ratio between the intensity at 405 nm with respect to that obtained
223 at 488 nm. For pseudocolor display, the ratio was coded on a spectral color scale ranging from
224 blue (fully reduced) to red (fully oxidized).
225
226 **Immunoblot analyses.**
227 Seven-day old 35S:GFP-ATG8a transgenic plants were transferred to MS liquid medium to carry
228 out the different treatments for 6 h. Then, total proteins were extracted. The extracts were
229 centrifuged at 10 000 g for 10 min, and equal amounts of proteins from the supernatants were
230 subjected to SDS-PAGE and immunoblotted with anti-GFP antibodies (Roche).
231
232 **Physiological parameters.**
233 Seven-day old Arabidopsis seedlings were exposed to the different treatments for 8 h and then
234 the production of H_2O_2 and O_2^-, quantum efficiency of PSII photochemistry (ΦPSII), as well as the

content of chlorophyll, soluble sugars and malondialdehyde (MDA) were analyzed. To assess H_2O_2 and O_2^- accumulation, leaves were stained with DAB and NBT, respectively. The staining was quantified by measuring the intensity per area using ImageJ. Quantum efficiency of PSII photochemistry (ΦPSII) was measured from the upper surface using a pulse amplitude modulated fluorometer (FMS2, Hansatech Instruments, Pentney King's Lynn, UK). Measurements were performed 6 h after the growth chamber lights were turned on. For chlorophyll, soluble sugars, and MDA analysis an alcoholic extract (1:2 w/v) was obtained from the samples. After centrifugation for 15 minutes at 12,000 *g*, the supernatant was diluted in ethanol, and the absorbance was measured at 654 nm using a spectrophotometer. Total chlorophylls were calculated following the method reported by (Wintermans M. and De Mots A., 1965). For soluble sugars determination, an aliquot of the sample was dried, and resuspended in a same volume of water. Then, the concentration of sugars was measured with anthrone (Fales, 1951) evaluating the absorbance at 620 nm using a glucose standard curve. For MDA determination, a volume of the supernatant was mixed with a same volume of a mixture of 0,5 % TBA and 20 % TCA, and incubated 20 minutes at 90 °C. The mixture was centrifuged at 12,000 *g* for 5 minutes, and the absorbance was measured at 532 and 600 nm (Heath and Packer, 1968).

RNA extraction and RT-qPCR

Samples were homogenized in a cold mortar with TRIzol Reagent, mixed for 1 min and incubated at room temperature for 5 min. Then, 0.2 ml chloroform per ml of TRIzol Reagent was added and incubated at room temperature for 3 min. After incubation, the samples were centrifuged at 12 000 *g* at 4 °C for 15 min and the aqueous phases were transferred to clean tubes. RNA was precipitated by adding 1 vol of isopropanol, incubated at room temperature for 10 min and centrifuged at 12 000 *g* at 4 °C for 15 min. The precipitate was washed with 70 % ethanol and the samples were centrifuged again at 12 000 *g* at 4 °C for 15 min. The precipitates were dried and resuspended in diethyl pyrocarbonate-treated water and its concentration was quantified using a NanoDrop 3300 spectrometer (Thermo Scientific). Purified RNA was treated with DNase I (Invitrogen) to remove genomic DNA, according to the manufacturer's instructions. DNA-free RNA (1 to 2.5 µg) was used with oligo(dT) for first strand cDNA synthesis using the Moloney murine leukemia virus reverse transcriptase (M-MLV RT, Promega) according to the manufacturer's instructions. For each primer pair, the presence of a unique product of the expected size was checked on ethidium bromide-stained agarose gels after PCR reactions. Absence of contaminant genomic DNA was confirmed in reactions with DNase-treated RNA as template. The qPCR reaction was performed using iQ Universal SYBR Green Supermix (Bio-Rad). Amplification of actin with the forward primer 5'-TTCAGATGCCCAGAAGTCTTG-3' and the reverse primer 5-GCGATACCTGAGAACATAGTGG-3 was used to normalize the amount of template cDNA. The specific primers pair employed for the detection of *Atg6* was forward primer 5'-AAAACTGGGAGAGCGATGAG-3' and reverse primer 5'-GATAGATGAGCCCTGCGTAG-3', for *Atg4a* was 5'-CCTTTGGAATCACTCGACCCATCT-3' and 5'-ATCCGCAAACCCGTAGTTGCTT-3', for *Atg7* was 5'-GCGGCTTTAGGTTTTGACAG-3' and 5'-GTTGGTCTAGAGTTCGATCAGTC -3', and for *Atg5* was 5'-TCCTGTTCGGTTGTATGTTCG-3' and 5'-CGCTCTGTCTCCCATAAACTC-3'. qPCR was performed in thermocycler iQ5 (Bio-Rad) at 59 °C with iQ SYBR Green Supermix (Bio-Rad), according to the manufacturer's instruction. Relative transcript abundance was calculated (Livak and Schmittgen, 2001).

Microarray analysis.

Publicly available microarray data were utilized to compare the expression of *Atg* genes under various conditions, including nitrogen deficiency (Rubin *et al.*, 2009), treatment with 10 mM

285 H_2O_2 for 24 h (Willems *et al.*, 2016), treatment with 10 µM MV for 2 h (Scarpeci *et al.*, 2008),
286 and the examination of *flu* mutant plants subjected to an 8-hour cycle of darkness followed by
287 2 h of light (Op Den Camp *et al.*, 2003). Expression values were normalized to the corresponding
288 control conditions employed in each study and were expressed as log_2 values.
289
290 **Statistical analyses.**
291 Data were analysed by analysis of variance (ANOVA) followed by the Di Rienzo–Guzman–
292 Casanoves (DGC) test model, a cluster-based method for identifying groups of non-
293 homogeneous means, using InfoStat software (Di Rienzo *et al.*, 2002).
294
295 **RESULTS**
296 **Methyl viologen and H_2O_2 oxidized the cytoplasmic redox state and inhibited autophagy in**
297 **Arabidopsis.**
298 To gain insights into the interplay between ROS and autophagy, *Arabidopsis thaliana*
299 seedlings were subjected to well-established autophagy-inducing conditions, namely nutritional
300 starvation and different abiotic stress treatments (Wang *et al.*, 2021), and both autophagy and
301 the cytoplasmic redox state were analyzed (Fig. 1). To monitor autophagy, 35S:GFP-ATG8a (GFP-
302 ATG8) transgenic plants were grown 7 days in MS solid medium, and then transferred to the
303 different treatments in MS liquid medium supplemented with 1 µM Concanamycin A (Conc A)
304 for a 16 hour-period. Conc A is a V-ATPase inhibitor widely used to prevent vacuole acidification,
305 thereby effectively inhibiting the lytic activity of vacuoles, thus allowing the detection of
306 autophagic bodies accumulation into the vacuoles through confocal microscopy (Fig. 1A and B).
307 Analysis of autophagic flux using Conc A better defines autophagic activity since the increase of
308 autophagosome number in the cytoplasm does not necessarily corresponds to an enhanced
309 autophagic activity (Robert *et al.*, 2021). When subjected to carbon and nitrogen starvation
310 treatments, epidermal cells showed a high accumulation of autophagic bodies in the vacuole
311 compared to control conditions, indicating an activated autophagic flux (Fig. 1A and B). Similarly,
312 saline and osmotic stress treatments resulted in comparable outcomes, with a significant
313 increase in autophagic bodies accumulating within the vacuoles (Fig. 1A and B). Besides, to
314 evaluate the cytoplasmic redox state *in vivo*, 7 days old transgenic plants expressing the cytosol-
315 targeted fluorescent redox sensors GFP2-Orp1 or GRX-roGFP2 were subjected to the different
316 treatments (Fig. 1). These transgenic plants express redox-sensitive green fluorescent proteins
317 (roGFPs) genetically fused to different redox-active proteins sensing H_2O_2 production and
318 GSH:GSSG, respectively (Aller *et al.*, 2013; Nietzel *et al.*, 2019). The relative intensity of the two
319 fluorescence excitation maxima depends on the redox state of the modified GFP, enabling
320 ratiometric measurements, independent of the probe concentration (Schwarzländer *et al.*,
321 2016). Notably, in epidermal cells of seedlings expressing either GFP2-Orp1 or GRX-roGFP2, no
322 changes in the cytoplasmic redox state were detected after 16 h of nutritional starvation, saline
323 or osmotic stress treatments compared to control conditions (Fig. 1A and C).
324 To further investigate the interplay between ROS and autophagy, oxidative stress
325 treatments using methyl viologen (MV) and H_2O_2 were conducted. MV is an herbicide that
326 generates O_2^- and H_2O_2 in the presence of light inside chloroplasts (Lascano *et al.*, 2012).
327 Previously, we evaluated different concentrations of MV and H_2O_2 (Fig. S1 and Fig. S2,
328 respectively). For the subsequent experiments, we used 1 µM MV since it was the lowest
329 concentration that showed effects on the cytoplasmic redox state (Fig. S1). Although oxidative
330 treatments have been reported as autophagy-inducing conditions in Arabidopsis and
331 Chlamydomonas (Xiong *et al.*, 2007*b*; Pérez-Pérez *et al.*, 2012*b*), no significant differences were

332 observed in the accumulation of autophagic bodies in the epidermal cells of seedlings treated
333 with MV or H_2O_2 compared to the control condition (Fig. 1A and B). Interestingly, contrary to the
334 observations under nutritional starvation, saline or osmotic stresses, these oxidative treatments
335 were able to significantly oxidize both biosensors indicating an increase in H_2O_2 levels and
336 GSSG:GSH ratio in the cytoplasm (Fig. 1A and C).
337

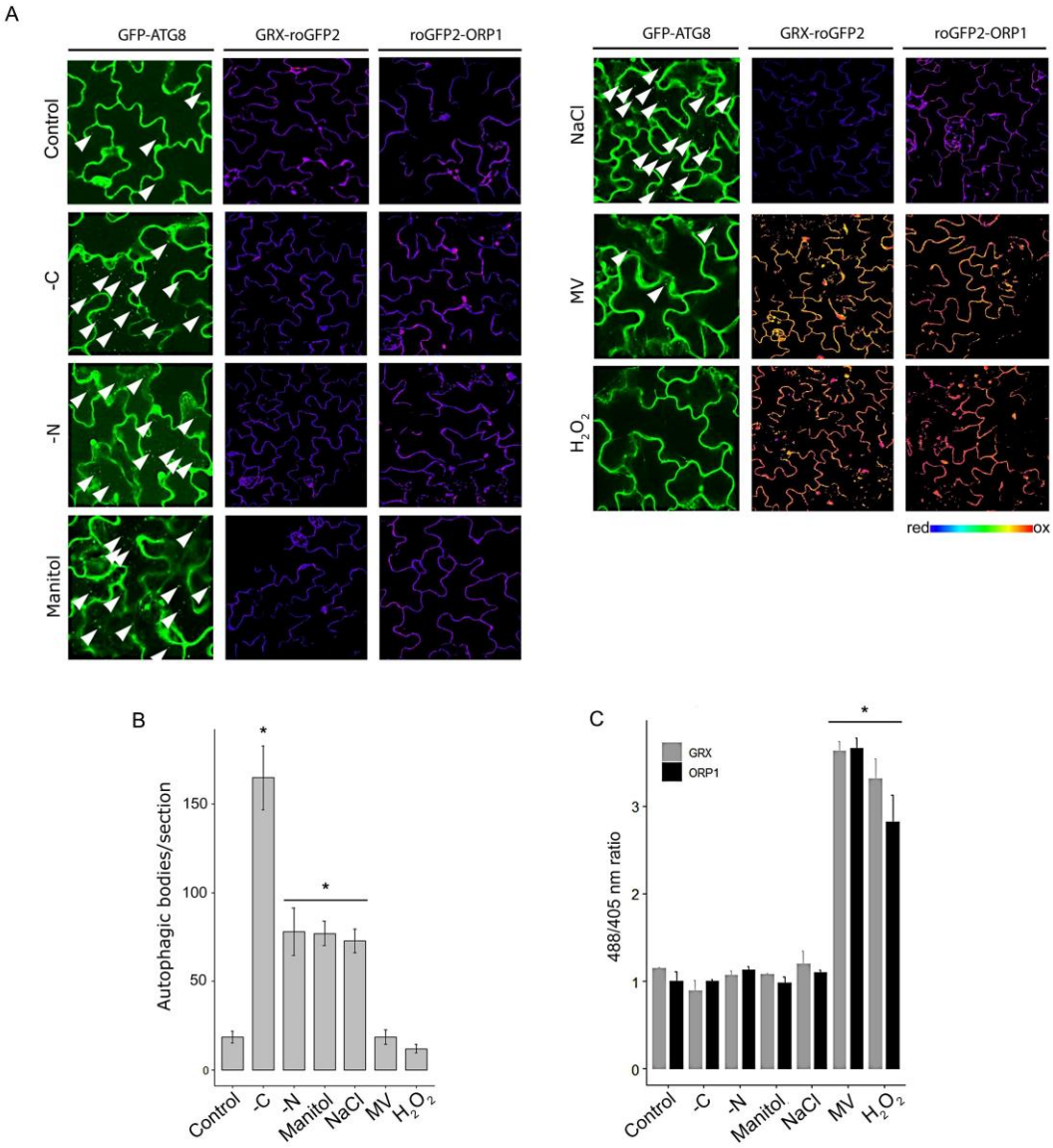

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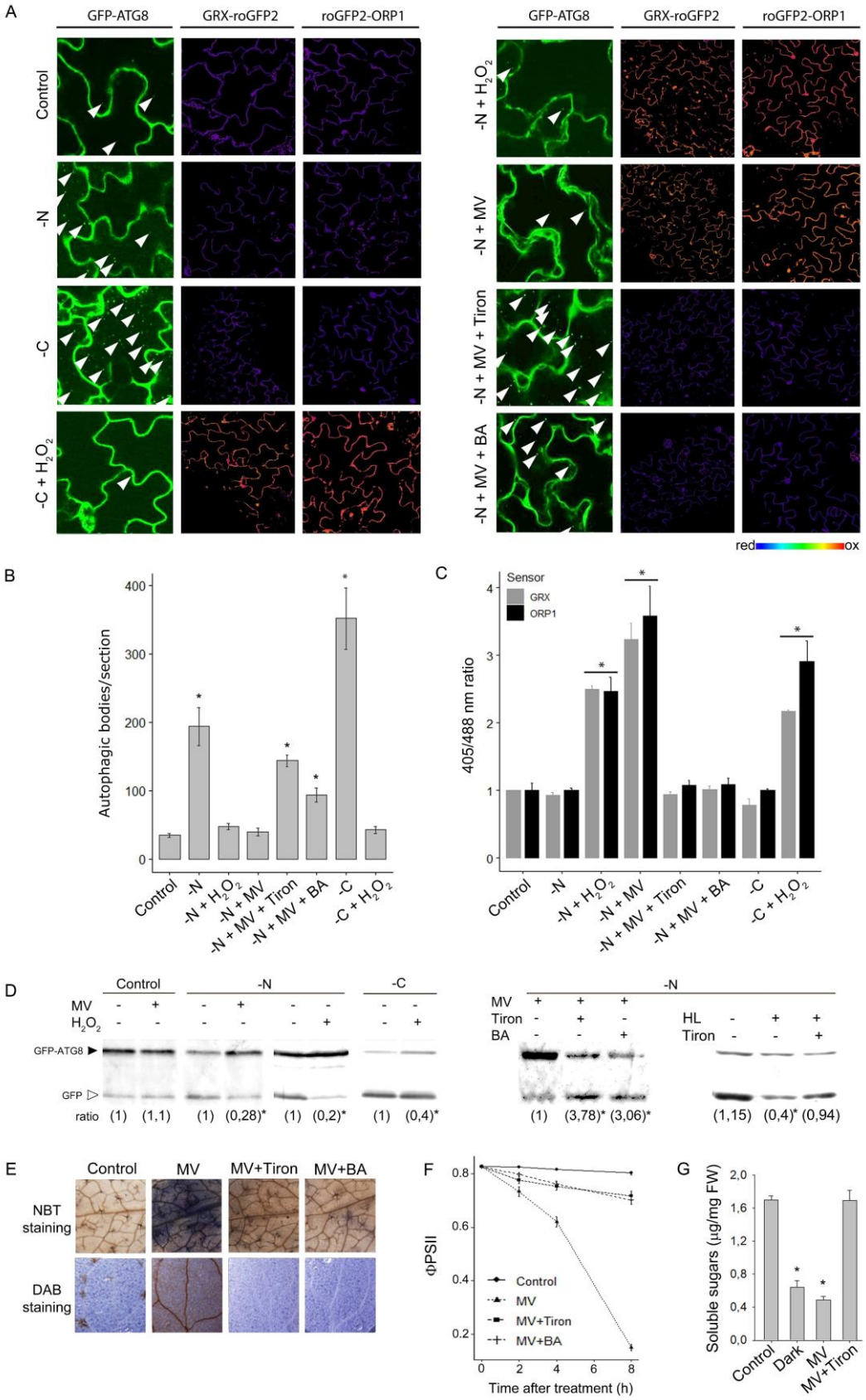
Figure 1: Autophagic flux and cytoplasmic redox state in *Arabidopsis thaliana* plants exposed to different abiotic stress conditions. (A-C) Seven-day old 35S:GFP-ATG8a transgenic plants and cytosol-targeted fluorescent redox sensors GFP2-Orp1 and GRX-roGFP2 expressing plants were exposed to different stress conditions in MS liquid medium. To induce autophagic bodies accumulation, MS liquid medium was supplemented with 1 µM Concanamycin A. (A) The stress treatments were performed for 16 h, followed by the evaluation of autophagic bodies accumulation (GFP-ATG8 columns) and the cytoplasmic redox state (GRX-roGFP and GFP2-Orp1 columns) by confocal microscopy. Arrowheads indicate autophagic bodies. GRX-roGFP and GFP2-Orp1 columns are ratiometric images. For pseudocolor display, the ratio was coded on a spectral color scale ranging from blue (fully reduced) to red (fully oxidized). Representative images are shown. The treatments included: MS with nitrogen (control), MS with nitrogen in darkness (-C), MS without nitrogen (-N), MS with nitrogen supplemented with 100 mM mannitol (Manitol), 50 mM NaCl (NaCl), 1 µM methyl viologen (MV) and 10 mM H_2O_2. (B) Average number of autophagic bodies accumulation per section. The average number of autophagic bodies was calculated from single-plane confocal images. Two sections from at least 7 plants were analyzed. (C) Average of cytoplasmic redox state expressed as the ratio of the fluorescence intensities measured at 488 nm and 405 nm (data expressed relative to the control condition). At least 6 plants were analyzed. Statistically significant differences are indicated by asterisks (*$P < 0.05$; error bars: means ± SEM, DGC test).

To further characterize the effects of oxidative stress on autophagy, treatments with MV and H_2O_2 were performed on 35S:GFP-ATG8a plants under autophagic inducing conditions, specifically nitrogen and carbon starvation (Fig. 2). Interestingly, treatments with MV and H_2O_2 reduced the accumulation of autophagic bodies after 16 h of starvation conditions (Fig. 2A and B). Furthermore, the inhibitory effects of the oxidative treatments on the autophagic flux were also confirmed by analyzing the degradation of the GFP-ATG8a fusion protein by western blot (Fig. 2D). The evaluation of autophagic flux through the analysis of the degradation of the GFP-ATG8 fusion protein by immunoblotting allowed us to examine the effects of oxidative stress on autophagy in shorter time periods (6 h, Fig. 2D). Next, to scavenge superoxide and peroxide, tiron and benzoic acid were co-administered with the MV treatment. The oxidative effects of MV on the autophagic flux (Fig. 2A, B and D) and the cytoplasmic redox state (Fig. 2B and C) were blocked in the presence of ROS scavengers. Similarly, photooxidative treatments showed an inhibitory effect on the autophagy flux, which was reversed in the presence of the superoxide radical scavenger, tiron (Fig. 2D). Moreover, both tiron and benzoic acid reduced the accumulation of H_2O_2 and O_2^- generated under oxidative stress, as indicated by DAB and NBT staining, respectively (Fig. 2E), as well as prevented photosynthetic damage caused by MV treatments, as measured by the quantum efficiency of photosystem II (ΦPSII) (Fig. 2F). Besides, the scavenging of O_2^- by the administration of tiron also prevented the decrease in soluble sugar levels that was observed in the MV treatments (Fig. 2G). Furthermore, the inhibitory effects of MV on autophagic flux and the oxidation of the GSH:GSSG sensor probe (GRX-roGFP2) were reversed when 10 mM dithiothreitol (DTT), a reducing agent for protein disulfide bonds, was added (Fig. S3). Nevertheless, DTT treatments exhibited slight effects on H_2O_2 production compared to tiron treatments (Fig. S3). These results emphasize that the effects of MV in plants are associated to its ability to promote the generation of ROS, contributing to a more oxidized cellular redox state and oxidative damage (Lascano *et al.*, 2012). It is noteworthy that the effect of tiron, benzoic acid, and DTT in restoring the autophagic flux in MV-treated plants suggested that H_2O_2 may inhibit autophagy, potentially by oxidizing disulfide bonds in key ATG proteins (Fig. S4 and S5) as demonstrated in recent studies in yeast, Chlamydomonas and animals (Pérez-Pérez *et al.*, 2014, 2016; Frudd *et al.*, 2018; Zheng *et al.*, 2020). In this regard, the inhibitory effect of H_2O_2 on autophagy was also observed in plants subjected to carbon or nitrogen starvation (Fig. 2A, B and D; Fig. S2).


A
GFP-ATG8
GRX-roGFP2
roGFP2-ORP1
Control
-N
-C
-C + H2O2
-N + H2O2
-N + MV
-N + MV + Tiron
-N + MV + BA
red
ox
B
Autophagic bodies/section
Control
-N
-N + H2O2
-N + MV
-N + MV + Tiron
-N + MV + BA
-C
-C + H2O2
C
Sensor
GRX
ORP1
405/488 nm ratio
D
Control
-N
-C
MV
H2O2
GFP-ATG8
GFP
ratio (1) (1,1) (1) (0,28)* (1) (0,2)* (1) (0,4)*
Tiron
BA
HL
(1) (3,78)* (3,06)* (1,15) (0,4)* (0,94)
E
Control
MV
MV+Tiron
MV+BA
NBT staining
DAB staining
F
ΦPSII
Control
MV
MV+Tiron
MV+BA
Time after treatment (h)
G
Soluble sugars (µg/mg FW)
Control
Dark
MV
MV+Tiron

375

Figure 2: Treatments with MV and H_2O_2 oxidized the cytoplasmic redox state and prevented autophagy induction in Arabidopsis. (A-C) Seven-day old 35S:GFP-ATG8a transgenic plants and cytosol-targeted fluorescent redox sensors GFP2-Orp1 and GRX-roGFP2 expressing plants were exposed to autophagy-inducing conditions and the effect of methyl viologen (MV) and H_2O_2 on autophagic flux and cytoplasmic redox state were analyzed. The stress treatments were performed for 16 h, followed by the evaluation of autophagic bodies accumulation (GFP-ATG8 columns) and the cytoplasmic redox state (GRX-roGFP and GFP2-Orp1 columns) by confocal microscopy. To induced autophagic bodies accumulation, MS liquid medium was supplemented with 1 µM Concanamycin A. Arrowheads indicate autophagic bodies. GRX-roGFP and GFP2-Orp1 columns are ratiometric images. For pseudocolor display, the ratio was coded on a spectral color scale ranging from blue (fully reduced) to red (fully oxidized). Representative images are shown. The treatments included: MS with nitrogen (control), MS with nitrogen in darkness (-C) supplemented with 10 mM H_2O_2 (-C + H_2O_2), MS without nitrogen (-N) supplemented with 1 µM methyl viologen (-N + MV) or 10 mM H_2O_2 (-N + H_2O_2). 10 mM Tiron and 10 mM Benzoic acid (BA) were used for ROS scavenging (-N + MV + Tiron and -N + MV + BA). (B) Average of autophagic bodies accumulation per section. The average of autophagic bodies number was calculated from single-plane confocal images. Two sections from at least 6 plants were analyzed. (C) Average of cytoplasmic redox state expressed as the ratio of the fluorescence intensities measured at 488 nm and 405 nm from single-plane confocal images (data expressed relative to control condition). At least 6 plants were analyzed. (D) Effects of oxidative treatments on the autophagic flux evaluated through the degradation of the GFP-ATG8a fusion protein by western blot. Seven-day old 35S:GFP-ATG8a transgenic Arabidopsis seedlings were exposed to the different treatments for 6 h. Total protein (40 µg) was subjected to immunoblot analysis with anti-GFP antibodies. The GFP-ATG8a fusion has a predicted molecular weight of approximately 40 kDa (closed arrowheads); free GFP has a predicted molecular weight of 27 kDa (open arrowheads). Representative images are shown. Ratio: the numbers in brackets denote the GFP/GFP-ATG8 ratio relativized to the condition without oxidative treatment. Three replicates were performed (more than fifty plants per replicate). HL: high light treatment (≈1000 µmoles m^{-2} s^{-1}). (E-G) Seven-day old Arabidopsis seedlings were exposed to 1 µM methyl viologen (MV), MV + 10 mM Tiron, MV + 10 mM BA, and control condition (without MV) for 8 h, and the different physiological parameters were analyzed. (E) Production of H_2O_2 and O_2^- revealed by *in situ* staining with 3',3'-diaininobenzidine (DAB) and nitroblue tetrazolium (NBT), respectively; (F) quantum efficiency of PSII photochemistry (ΦPSII), and (G) Soluble sugar content. Asterisks indicate statistically significant differences (*$P < 0.05$; error bars: means ± SEM, DGC test)

Autophagy is induced in the recovery phase after oxidative stress in Arabidopsis.

Previous reports indicated that autophagy is involved in degrading oxidized proteins and organelles in response to oxidative stress (Xiong *et al.*, 2007*b*; Shibata *et al.*, 2013; Izumi *et al.*, 2017; Oikawa *et al.*, 2022). However, our experimental results demonstrate that oxidative stress treatments inhibit autophagy, as illustrated in Figure 2. Considering these findings, we aimed to investigate the autophagic activity following oxidative stress treatments by evaluating the autophagic flux in Arabidopsis plants during the recovery phase. With this purpose, 7-day-old Arabidopsis seedlings were subjected to a brief exposure of 10 mM H_2O_2 or high light intensity (≈1000 µmoles m^{-2} s^{-1}) for 4 hours, followed by transfer to H_2O_2-free MS liquid medium for a 20-hour recovery period. Additionally, a set of plants were incubated in 10 mM H_2O_2 or to control conditions (H_2O_2-free MS liquid medium) for 24 h. This experimental design aimed to elucidate the dynamic changes in autophagy following oxidative stress and determine whether autophagic activity is modulated during the recovery phase. The results showed that autophagic flux was inhibited in plants incubated 24 h with H_2O_2 compared to control conditions (Fig. 3A, B and D). However, autophagic flux was not only recovered but also induced during the recovery period after the stress treatments surpassing the levels observed in plants subjected to control conditions (Fig. 3A, B and D). Likewise, after the 20-hour recovery period, the redox biosensors returned to their reduced state, indicating both a successful cytoplasmic redox recovery, and a clear correlation between reduced cytoplasmic redox state and autophagic flux (Fig. 3A and C).

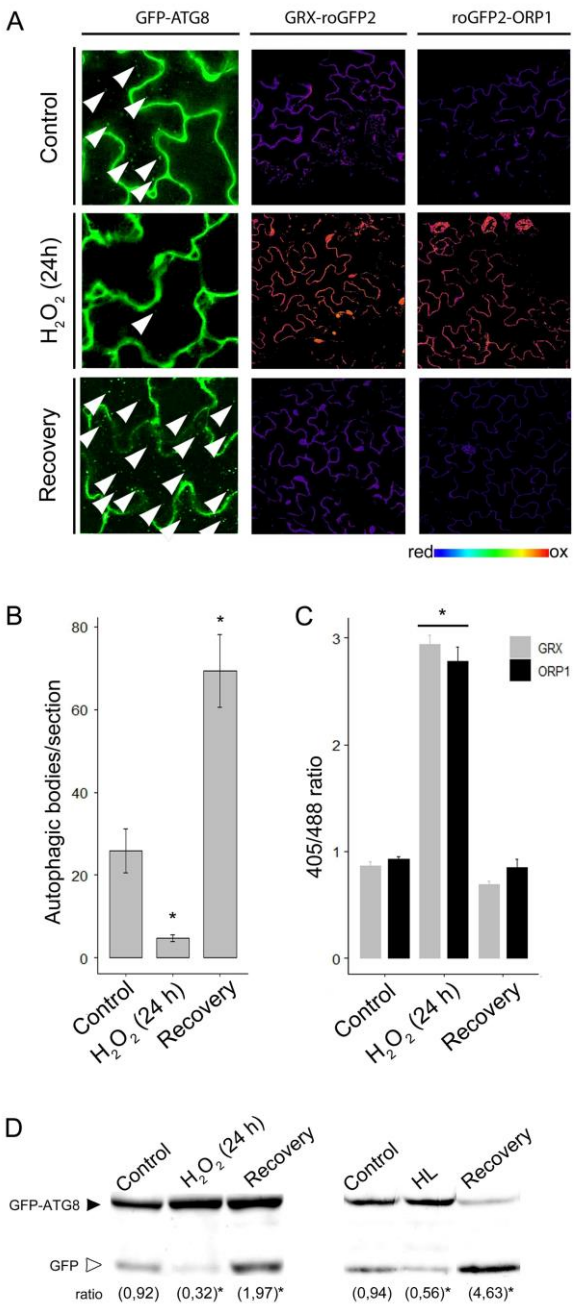

A
GFP-ATG8
GRX-roGFP2
roGFP2-ORP1
Control
H2O2 (24h)
Recovery
red
ox
B
Autophagic bodies/section
Control
H2O2 (24 h)
Recovery
C
405/488 ratio
GRX
ORP1
Control
H2O2 (24 h)
Recovery
D
Control
H2O2 (24 h)
Recovery
GFP-ATG8
GFP
ratio (0,92) (0,32)* (1,97)*
Control
HL
Recovery
(0,94) (0,56)* (4,63)*

Figure 3: Autophagy is induced after oxidative stress treatments in Arabidopsis. (A-D) Seven-day old transgenic plants expressing 35S:GFP-ATG8a and cytosol-targeted fluorescent redox sensors GFP2-Orp1 and GRX-roGFP2 were subjected to a brief exposure of oxidative stress, either 10 mM H_2O_2 or high light intensity (≈1000 µmoles m^{-2} s^{-1}), for 4 h. Subsequently they were transferred to MS liquid medium for a 20-hour recovery period under continuous light (120 µmoles m^{-2} s^{-1}) (recovery). Control plants were incubated under continuous light (120 µmoles m^{-2} s^{-1}) in H_2O_2-free MS liquid medium for 24 h. Stressed plants were incubated in MS supplemented with 10 mM H_2O_2 or high light intensity (≈1000 µmoles m^{-2} s^{-1}) for 24 h. (A) To induce accumulation of autophagic bodies, MS liquid medium was supplemented with 1 µM Concanamycin A. Arrowheads indicate autophagic bodies. GRX-roGFP and GFP2-Orp1 columns represent ratiometric images, with pseudocolor display indicating the ratio on a spectral color scale ranging from blue (fully reduced) to red (fully oxidized). Representative images are shown. (B) Average number of autophagic bodies accumulation per section. The average number of autophagic bodies was calculated from single-plane confocal images. Two sections from at least 6 plants were analyzed. (C) Average cytoplasmic redox state expressed as the ratio of the fluorescence intensities measured at 488 nm and 405 nm from single-plane confocal images (data are expressed relative to the control condition). At least 6 plants were analyzed. Asterisks indicate statistically significant differences (*$P < 0.05$; error bars: means ± SEM, DGC test). (D) Autophagic flux evaluated through the degradation of the GFP-ATG8a fusion protein by western blot. Total protein (40 µg) was subjected to immunoblot analysis with anti-GFP antibodies. The GFP-ATG8a fusion has a predicted molecular weight of approximately 40 kDa (closed arrowheads), while free GFP has a predicted molecular weight of 27 kDa (open arrowheads). Representative images are shown. The numbers in brackets denote the GFP/GFP-ATG8 ratio relativized to the control condition. Three replicates were performed (n > 50 plants per replicate). Asterisks indicate statistically significant differences (*$P < 0.05$; error bars: means ± SEM, DGC test).

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434
435 **Singlet oxygen induced autophagic flux but did not affect the cytoplasmic redox state.**
436 Singlet oxygen ($^1O_2$) is mainly produced in the chloroplast, and its targets are
437 predominantly confined inside this organelle due to the specific physicochemical characteristic
438 of this ROS (Dmitrieva *et al.*, 2020). To investigate the effects of $^1O_2$ on the autophagic activity,
439 GFP-ATG8a seedlings were incubated with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)
440 (Fig. 4), an herbicide that blocks plastoquinone reduction promoting $^1O_2$ generation at the PSII
441 (Flors *et al.*, 2006), and the accumulation of autophagic bodies and cytoplasmic redox state were
442 analyzed after 16 h (Fig. 4A-C). Additionally, treatments with methylene blue (MB), a type-II
443 photosensitizer able to generate $^1O_2$ in leaves under visible light, with minor effects on
444 photosynthesis (Fig. 4E), were also performed. Treatments with these chemicals increased the
445 accumulation of autophagic bodies but did not show statistically significant effects on the redox
446 state of the cytoplasm in Arabidopsis cells compared to control treatment (Fig. 4A and C) (Fig.
447 4A and B). The autophagic flux-inducing effect of DCMU and MB was also evidenced after 6
448 hours of treatments through the analysis of GFP-ATG8 degradation by immunoblotting (Fig. 4D).
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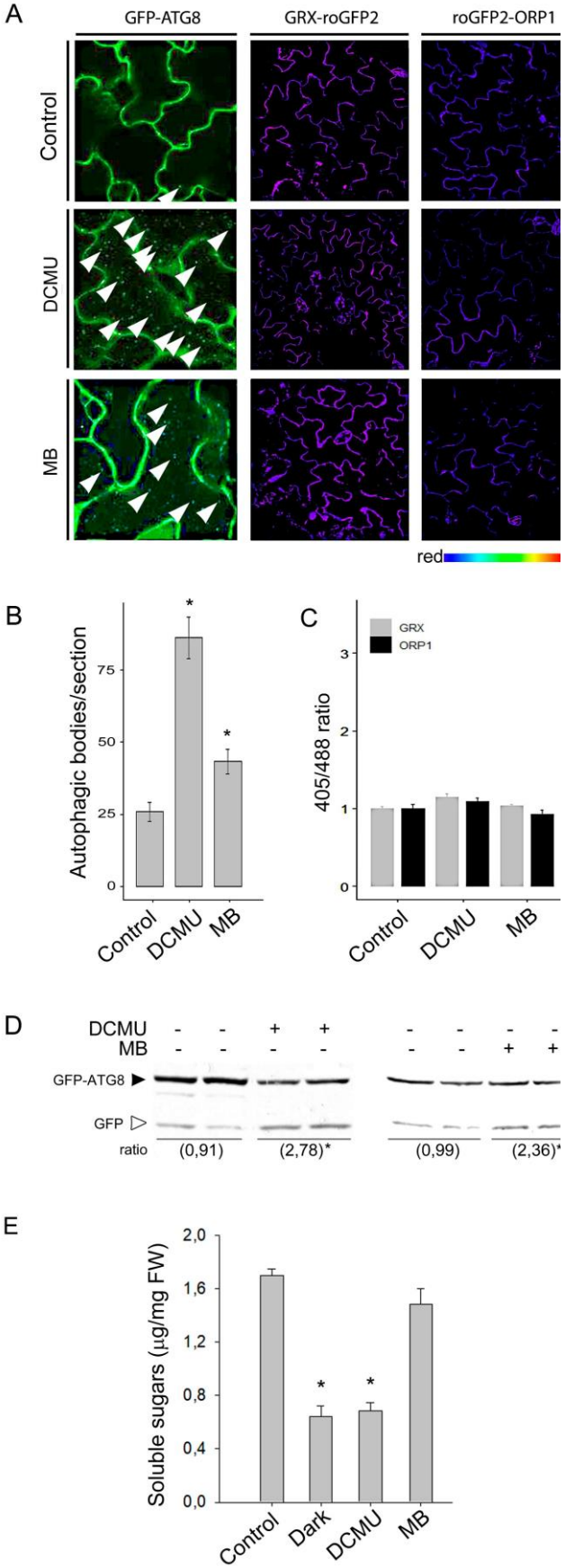
A
GFP-ATG8
GRX-roGFP2
roGFP2-ORP1
Control
DCMU
MB
red
B
Autophagic bodies/section
75
50
25
0
*
*
Control
DCMU
MB
C
GRX
ORP1
405/488 ratio
3
2
1
0
Control
DCMU
MB
D
DCMU - - + + - - - -
MB - - - - - - + +
GFP-ATG8
GFP
ratio (0,91) (2,78)* (0,99) (2,36)*
E
Soluble sugars (µg/mg FW)
2,0
1,6
1,2
0,8
0,4
0,0
*
*
Control
Dark
DCMU
MB

Figure 4: DCMU and methylene blue treatments induced autophagic flux in Arabidopsis. (A-D) Seven-day old transgenic plants expressing 35S:GFP-ATG8a and cytosol-targeted fluorescent redox sensors GFP2-Orp1 and GRX-roGFP2 were incubated in MS medium supplemented with either 20 µM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) or 20 µM methylene blue (MB), and the autophagic flux and cytoplasmic redox state were evaluated. (A) Autophagic bodies accumulation and cytoplasmic redox state after 16 h of treatment. To induce autophagic bodies accumulation, MS liquid medium was supplemented with 1 µM Concanamycin A. Autophagic bodies are indicated by arrowheads. The GRX-roGFP and GFP2-Orp1 columns represent ratiometric images. For pseudo color display, the ratio was coded using a spectral color scale ranging from blue (fully reduced) to red (fully oxidized). Representative images are shown. (B) Average number of autophagic bodies per section. The average number of autophagic bodies was calculated from single-plane confocal images. Two sections from at least 5 plants were analyzed. (C) Average cytoplasmic redox state expressed as the ratio of the fluorescence intensities measured at 488 nm and 405 nm from single-plane confocal images (data expressed relative to control condition). Analysis was performed on at least 4 plants. Asterisks indicate statistically significant differences (*$P < 0.05$; error bars: means ± SEM, DGC test). (D) Evaluation of the effects of DCMU and MB on autophagic flux through degradation of the GFP-ATG8a fusion protein by western blot. Seven-day old transgenic Arabidopsis seedlings expressing 35S:GFP-ATG8a were exposed to the different treatments for 6 h. Total protein (40 µg) was subjected to immunoblot analysis with anti-GFP antibodies. The GFP-ATG8a fusion has a predicted molecular weight of approximately 40 kDa (closed arrowheads), while free GFP has a predicted molecular weight of 27 kDa (open arrowheads). Representative images are shown. The numbers in brackets denote the GFP/GFP-ATG8 ratio relative to the control condition. Four replicates were performed with more than fifty plants per replicate. (E) Seven-day old Arabidopsis seedlings were exposed to the different treatments for 6 h and soluble sugar content was analyzed. FW: fresh weight. Asterisks indicate statistically significant differences (*$P < 0.05$; error bars: means ± SEM, DGC test).

To further investigate the effects of $^1O_2$ on the autophagic process and the cytoplasmic redox state, autophagy reporter- and redox sensor-transgenic plants were crossed with widely used endogenously chloroplast $^1O_2$-hyperproducing *Arabidopsis* mutant plants: fluorescent (*flu*) and two different lines of ferrochelatase 2 (*fc2*), *fc2.1* which presents reduced expression levels of *Fc2*, and the null mutant *fc2.2*. These mutant plants accumulate different photosensitizing intermediates of chlorophyll biosynthesis in the dark, which, upon light exposure lead to an exacerbated production of $^1O_2$ (Flors *et al.*, 2006). The *flu* mutant plants accumulate protochlorophyllide (Meskauskiene *et al.*, 2001), while mutations in plastid ferrochelatase 2 lead to the accumulation of protoporphyrin IX (Scharfenberg *et al.*, 2015). Initially, wild type, *flu* and *fc2* mutant plants were grown 7 days under continuous light conditions (control conditions), and several growth and physiological parameters were evaluated (Fig. S6). Interestingly, the *fc2* mutants showed arrested root growth, reduced chlorophyll levels, increased levels of MDA and reduced levels of soluble sugars compared to wild type, while no significant differences were observed between wild type and *flu* mutant (Fig. S6). Likewise, the cytoplasmic redox state in *fc2* plants was significantly more oxidized than that in the wild type and *flu* backgrounds (Fig. S6, Fig. 5). Furthermore, and consistent with a previous study (Lemke *et al.*, 2021), the autophagic flux was induced in *fc2* mutants plants compared to wild type and *flu* mutant under continuous light conditions (Fig. S6). Hence, considering the altered phenotypes observed in *fc2* mutants even under control conditions, we used the *flu* mutants for subsequent analysis.

flu/GFP-ATG8a plants were grown under continuous light conditions for 7 days, transferred to darkness for 8 h, and then re-exposed to light in the presence of Conc A for another 8 h. After the light/dark/light cycling conditions, a significantly increased autophagic flux was observed in *flu*/GFP-ATG8a compared to Col-0/GFP-ATG8 plants. However, no statistically significant differences were observed among these genotypes under continuous

light conditions (Fig. 5A, B and D). Besides, the cytoplasmic redox state was monitored under control conditions as well as after a period of darkness by analyzing the fluorescent redox sensor roGFP2-ORP1 expressed in the wild type and *flu* backgrounds. Although a slight tendency towards a more oxidized state was observed in the cytoplasm of *flu* mutant plants once re-exposed to light, the differences were not statistically significant (Fig. 5C). Thus, in contrast to H_2O_2 and MV treatments, $^1O_2$ increased the autophagic flux and did not oxidize the cytoplasmic redox state, indicating differential effects on the autophagic activity depending on the ROS nature and their effects on the cytoplasmic redox state. Moreover, the transcript levels of *Atg4*, *Atg5*, *Atg6* and *Atg7* were analyzed by RTq-PCR in *flu* mutant after 8 h of light re-exposure. However, no statistically significant differences were observed in the expression level of the analyzed genes between wild type and *flu* mutant neither under control conditions nor after cycling conditions (Fig. S7).

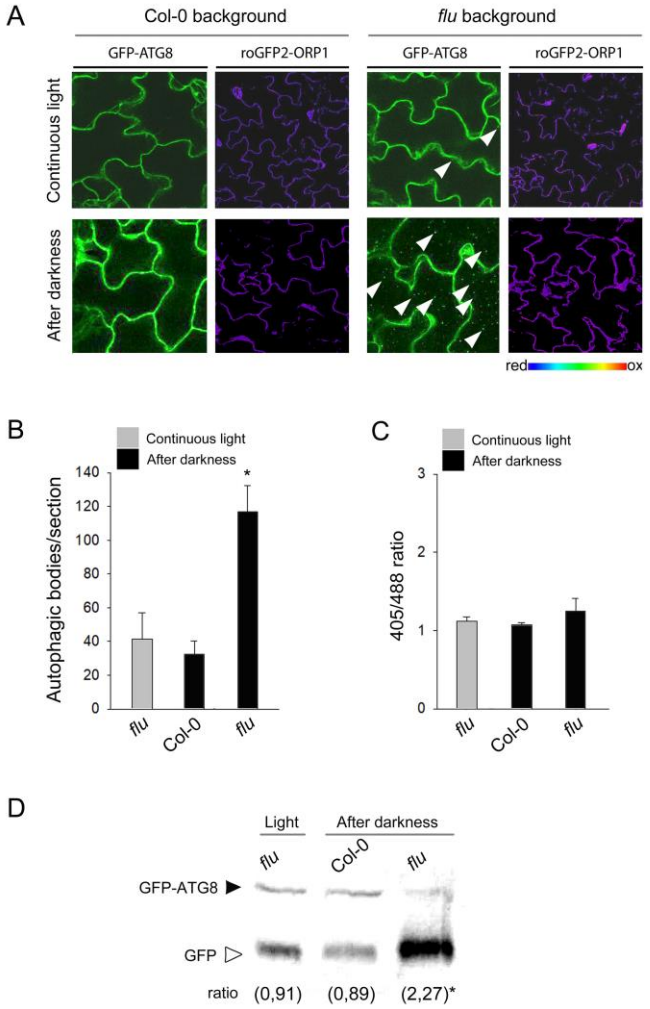

Figure 5: Autophagic flux is induced in conditionally chloroplast $_1O_2$-hyperproducing *flu* mutant Arabidopsis plants. (A-C) Seven-day old wildtype (Col-0) and *flu* mutant plants expressing 35S:GFP-ATG8a or GFP2-Orp1 were transferred to darkness for 8 h, followed by re-exposure to light. Control plants were maintained under continuous light conditions. After 8 h, the autophagic flux and cytoplasmic redox state were analyzed. (A) Autophagic bodies accumulation and cytoplasmic redox state. To induce autophagic bodies accumulation, the MS liquid medium was supplemented with 1 µM Concanamycin A. The GFP2-Orp1 columns represent ratiometric images. For pseudocolor display, the ratio was coded on a spectral color scale ranging from blue (fully reduced) to red (fully oxidized). Representative images are shown with arrowheads indicating autophagic bodies. (B) Average number of autophagic bodies accumulation per section. The average number of autophagic bodies was calculated from single-plane confocal images. Two different sections from at least 6 plants were analyzed. (C) Average cytoplasmic redox state expressed as the ratio of the fluorescence intensities measured at 488 nm and 405 nm from single-plane confocal images (data are expressed relative to Col-0 plants under control condition). At least 4 plants were analyzed. Asterisks indicate statistically significant differences (*$P < 0.05$; error bars: means ± SEM, DGC test). (D) Evaluation of autophagic flux through the degradation of the GFP-ATG8a fusion protein by western blot. Seven-day old wildtype (Col-0) and *flu* mutant plants expressing 35S:GFP-ATG8a were transferred to darkness for 8 h and then re-exposed to light for 6 h (after darkness). Continuous light was used as a control (light). Total protein (40 µg) was subjected to immunoblot analysis with anti-GFP antibodies. The GFP-ATG8a fusion has a predicted molecular weight of approximately 40 kDa (closed arrowheads); while free GFP has a predicted molecular weight of 27 kDa (open arrowheads). Representative images are shown. The numbers in brackets denote the GFP/GFP-ATG8 ratio relative to *flu* mutans under control condition. Three replicates were performed (more than fifty plants per replicate). Asterisks indicate statistically

551
552
553 **DISCUSSION**

554 Plants, as aerobic and sessile organisms, respond to environmental stresses by
555 increasing the production of reactive oxygen species (ROS), which, due to their high reactivity,
556 fulfill dual roles by acting both as agents of damage and as signaling molecules. Consequently,
557 ROS are universally implicated in plant stress symptomatology, and also play a pivotal role in
558 modulating plant growth, development, and cell death (Apel and Hirt, 2004; Trippi *et al.*, 2009).
559 Similarly, a multitude of stress conditions in plants have been demonstrated to initiate or
560 enhance autophagy (Bassham *et al.*, 2006; Liu *et al.*, 2009; Signorelli *et al.*, 2019; Su *et al.*, 2020).
561 Moreover, defects in antioxidant proteins in Arabidopsis and Chlamydomonas contribute to
562 uncontrolled ROS bursts and autophagy induction (Pérez-Pérez *et al.*, 2012*a*; Shibata *et al.*,
563 2013). While the concurrent induction of ROS production and autophagy activation suggests a
564 positive correlation between these cellular processes, certain molecular mechanisms have been
565 reported in animal cells, yeast, and Chlamydomonas, indicating that ROS could potentially exert
566 a negative influence on autophagy (Pérez-Pérez *et al.*, 2014, 2016; Frudd *et al.*, 2018). However,
567 direct evidence of ROS-mediated inhibition of autophagy remains scarce in plants. One of the
568 fundamental unanswered queries is whether a particular type of ROS functions as the primary
569 messenger, if any, to induce autophagy. Our findings point in that direction, highlighting the
570 differential effects of ROS on autophagy and suggesting that, under specific stress conditions
571 leading to a highly oxidized cytoplasmic state, the autophagic process may be suppressed.

572 In this study, MV was used to specifically generate O_2^- and H_2O_2 in the chloroplast.
573 Previous studies have reported the formation of autophagosomes in root cells following
574 incubation with MV (Xiong *et al.*, 2007*b*). Nevertheless, our findings differ from these outcomes.
575 It is important to acknowledge that certain variations in experimental and methodological
576 approaches between our research and the prior studies may have a considerable impact on the
577 interpretation of the outcomes. In the earlier studies, MV treatments were administered on
578 solid MS media, subjecting plants to 10 µM MV for a duration of two days, and the observations

579 were made in roots (heterotrophic tissues where MV produces O_2^- in mitochondria). Conversely,
580 in our study, MS liquid media was supplemented with 1 µM MV, and leaves were analyzed
581 (tissues where MV generates O_2^- in chloroplast). Furthermore, a critical distinction lies in the
582 method employed for quantifying autophagosomes. Previous investigations employed
583 monodansylcadaverine (MDC) staining to identify acidic compartments. However, experiments
584 involving autophagy-deficient *atg* mutants, as well as co-localization analysis in GFP-ATG8
585 plants, indicated that the MDC-stained structures do not clearly manifest as autophagosomes
586 and/or autolysosomes (Merkulova et al., 2014). In contrast, we used GFP-ATG8 plants for
587 autophagy analysis, which is a more reliable and widely accepted method for identifying
588 autophagosomes.

589 The relationship between autophagy and ROS is intricate, and our findings revealed that
590 different types of ROS, even when generated in the same organelle, can have different effects
591 on the autophagic process. We observed that under conditions of nutritional starvation, which
592 are canonical inducers of autophagy, oxidative treatments had an inhibitory effect on the
593 autophagic flux. This implies that the comparatively limited autophagosome biogenesis rate
594 under optimal growth conditions could potentially account for the absence of observable
595 inhibitory effects of ROS on autophagy in prior *in vivo* plant studies. Opposite to the inhibitory
596 effects of H_2O_2 on the autophagic flux, the specific generation of $^1O_2$ in the *flu* mutant plants, as
597 well as treatments with DCMU and MB, increased the autophagic activity. These findings are
598 consistent with previous research demonstrating that treatments with norflurazon, an inhibitor
599 of carotenoid biosynthesis, triggers autophagy in *Chlamydomonas reinhardtii* (Pérez-Pérez *et al.*,
600 2016). Similarly to what is observed in *flu* mutants (Flors *et al.*, 2006), norflurazon treatments
601 lead to perturbations in plastid homeostasis that increase the photosensitizing activity of
602 tetrapyrroles, which can transfer the excitation energy to the oxygen molecule, thereby
603 generating $^1O_2$ (Kim and Apel, 2013). With a shorter half-life and inability to cross membranes,
604 the activity of $^1O_2$ is confined to the thylakoid membrane of chloroplasts (Dmitrieva *et al.*, 2020).
605 These characteristics provide potential explanations for the absence of observable effects on
606 the cytoplasmic redox state in treatments involving DCMU, MB, and *flu* mutant plants.
607 Conversely, treatments with MV and H_2O_2 highly oxidized the cytoplasmic redox state. These
608 results establish a clear correlation between the cytoplasmic redox state of the cytoplasm and
609 autophagic activity.

610 The observation that the inhibition of autophagic flux and the oxidation of the
611 cytoplasmic redox state in MV treatments can be prevented by ROS scavengers such as tiron or
612 benzoic acid, as well as by treatment with the disulfide bonds reductant DTT, strengthens the
613 notion that H_2O_2 inhibits autophagy, but also suggests that protein oxidation in the cytoplasm
614 may be involved in this response. Notably, it has been proposed that proteins are the primary
615 targets of reactive oxidants and other radicals *in vivo* (Davies, 2005). At the molecular level, the
616 activity of certain ATG proteins has been shown to be regulated by redox changes. For instance,
617 ATG4, a protein involved in the cleavage of ATG8 for its lipidation and de-lipidation, is inhibited
618 by H_2O_2 in yeast and the green alga *Chlamydomonas reinhardtii* (Pérez-Pérez *et al.*, 2014, 2016).
619 Conversely, reducing agents such as DTT can restore the activity of ATG4 (Pérez-Pérez *et al.*,
620 2014, 2016). Furthermore, it has been reported in animal cells that oxidation of ATG3 and ATG7
621 leads to the inhibition of autophagy (Frudd *et al.*, 2018). Although the inhibition of autophagy
622 due to oxidations in ATG3 and ATG7 has not been tested in plants, the conservation of amino
623 acid sequence between the isoforms from yeast, human cells, and Arabidopsis (Fig. S4 and S5)
624 suggest that this type of regulation could also occur in plants.

625 The complexity of the relationship between autophagy and ROS becomes even more
626 complex when considering that this catabolic process is involved in the degradation of proteins

and oxidized organelles, which are primary sources of ROS production. The increased autophagic flux observed during the recovery period after exposure to oxidative stress, accompanied by the restoration of a reduced redox state in the cytoplasm, indicated that the inhibition of autophagy by ROS is transient. By assessing the autophagic flux, we aimed to gain insights into the role of autophagy in cellular adaptation to oxidative stress and its potential contribution to stress recovery mechanisms. Our results suggest that autophagy induction once the oxidative stress has subsided is associated with the intracellular clearance of cytoplasmic components damaged by oxidative stress, as indicated by the experiments with the antioxidants tiron and benzoic acid, which controlled ROS production and prevented cell injury. These observations highlight the dynamic nature of autophagy in response to oxidative stress and its subsequent recovery, suggesting a complex regulatory mechanism in Arabidopsis.

In summary, our work provides a novel perspective on the relationship between ROS and autophagy. The fact that the generation of O_2^-/H_2O_2 and $^1O_2$ have different effects on autophagy imply that the term "ROS" encompasses molecules with the ability to modulate this process in distinct ways. Further studies are needed to unravel the molecular mechanisms underlying the differential modulation of autophagy by ROS, being ATG4, ATG3 and ATG7 possible targets to be oxidatively regulated.

SUPPLEMENTARY DATA

Supplementary Figure 1: Assessment of different MV concentrations.
Supplementary Figure 2: Assessment of different H_2O_2 concentrations.
Supplementary Figure 3: DTT reversed the effects of MV treatments.
Supplementary Figure 4: Sequence alignment of ATG4 proteases from different organisms.
Supplementary Figure 5: Sequence alignment of ATG3 and ATG7 proteins from different organisms.
Supplementary Figure 6: Characterization of *fc2* and *flu* mutant plants under continuous light conditions.
Supplementary Figure 7: Transcript level of *Atg* genes in Arabidopsis plants subjected to different oxidative treatments.

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AUTHOR CONTRIBUTION:

GR and RL: conceptualization; GR, and AE: investigation; GR, AE, LS and RL: formal analysis; GR, LS, and RL: resources; GR and AE: writing - original draft; GR, AE, LS, and RL: writing - review & editing; GR and AE: visualization; GR and RL: supervision; GR and RL: funding acquisition.

CONFLICT OF INTEREST

No conflict of interest declared.

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677 (UNC) (RL).
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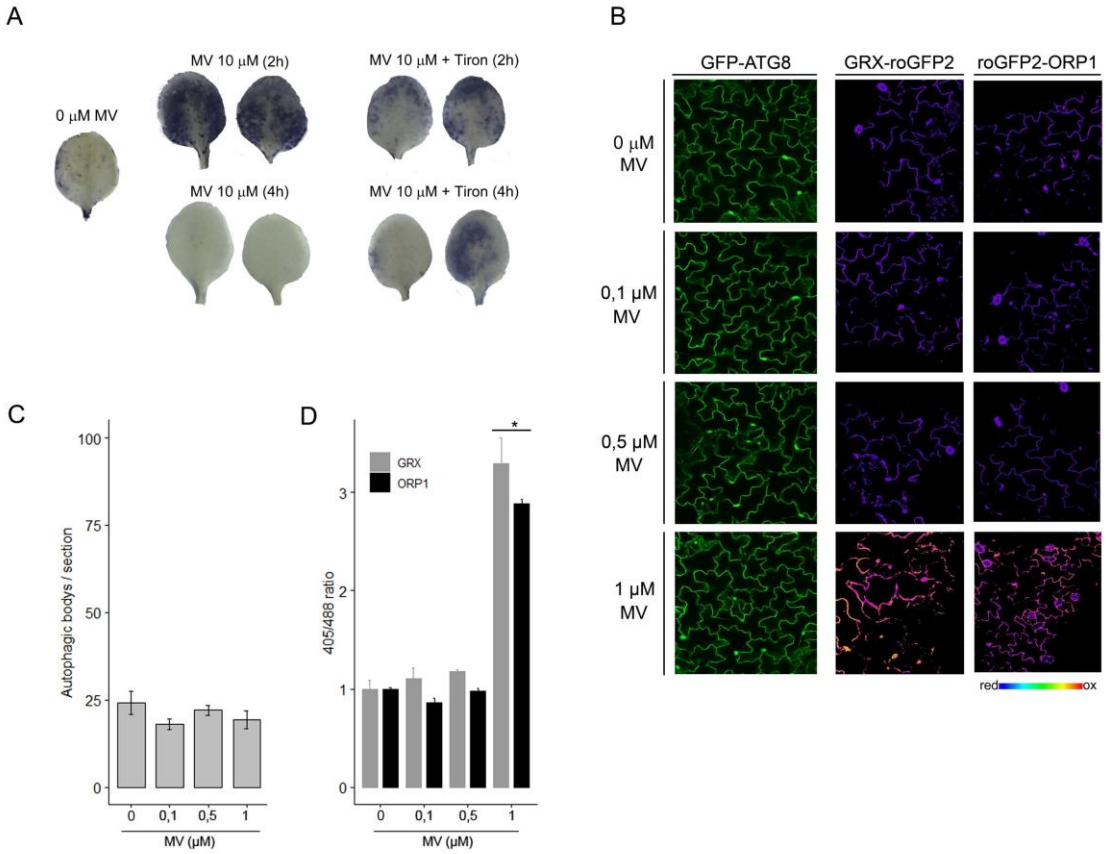
870 **Supplementary Figure 1**

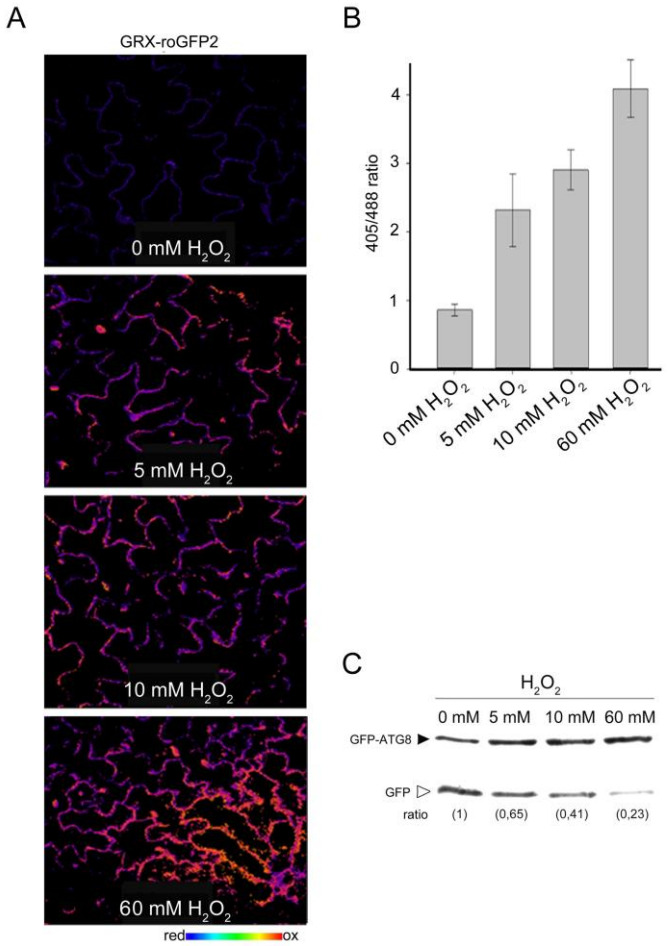
871



872 **Supplementary Figure 1:** (A) Effects of 10 µM MV on ROS generation. After 2 h, MV-treated
873 plants exhibit a significant accumulation of ROS. The co-administration of tiron led to a reduction
874 in ROS generation. After 4 h, plants treated with MV did not show ROS production, indicating
875 cell death. However, co-administration of tiron conferred resistance to the oxidative treatment,
876 as evidenced by the observed ROS production. (B-D) Autophagic flux and cytoplasmic redox state
877 in Arabidopsis plants exposed to different MV concentrations. Seven-day old 35S:GFP-ATG8a
878 transgenic plants and cytosol-targeted fluorescent redox sensors GFP2-Orp1 and GRX-roGFP2
879 expressing plants were exposed to different MV concentrations. To induce autophagic bodies
880 accumulation, MS liquid medium was supplemented with 1 µM Concanamycin A. The stress
881 treatments were performed for 16 h, followed by the evaluation of autophagic bodies
882 accumulation (GFP-ATG8 columns) and the cytoplasmic redox state (GRX-roGFP and GFP2-Orp1
883 columns) by confocal microscopy. GRX-roGFP and GFP2-Orp1 columns are ratiometric images.
884 For pseudocolor display, the ratio was coded on a spectral color scale ranging from blue (fully
885 reduced) to red (fully oxidized). Representative images are shown. (C) Average of autophagic
886 bodies accumulation per section. The average of autophagic bodies number was calculated from
887 single-plane confocal images. Two sections from at least 3 plants were analyzed. (D) Average of
888 cytoplasmic redox state expressed as the ratio of the fluorescence intensities measured at 488
889 and 405 (data expressed relative to 0 µM MV). At least 3 plants were analyzed.

890

891 **Supplementary Figure 2**



892

893 **Supplementary Figure 2:** (A-B) Cytoplasmic oxidation of the GSH:GSSG sensor probe (GRX-
894 roGFP2) in Arabidopsis plants exposed to different H_2O_2 concentrations. Seven-day old GRX-
895 roGFP2 expressing plants were exposed to different H_2O_2 concentrations. The stress treatments
896 were performed for 6 h, followed by the evaluation of the cytoplasmic redox state (GRX-roGFP)
897 by confocal microscopy. For pseudocolor display, the ratio was coded on a spectral color scale
898 ranging from blue (fully reduced) to red (fully oxidized). Representative images are shown. (B)
899 Average of cytoplasmic redox state expressed as the ratio of the fluorescence intensities
900 measured at 488 and 405 (data expressed relative to 0 µM H_2O_2). At least 3 plants were analyzed.
901 (C) Autophagic flux evaluated through the degradation of the GFP-ATG8a fusion protein by
902 western blot. Seven-day old 35S:GFP-ATG8a expressing plants were exposed to different H_2O_2
903 concentrations. Total protein (40 µg) was subjected to immunoblot analysis with anti-GFP
904 antibodies. The GFP-ATG8a fusion has a predicted molecular weight of approximately 40 kDa
905 (closed arrowheads); free GFP has a predicted molecular weight of 27 kDa (open arrowheads).
906 Representative images are shown. Ratio: the numbers in brackets denote the GFP/GFP-ATG8
907 ratio relativized to the condition without oxidative treatment (to 0 µM H_2O_2).
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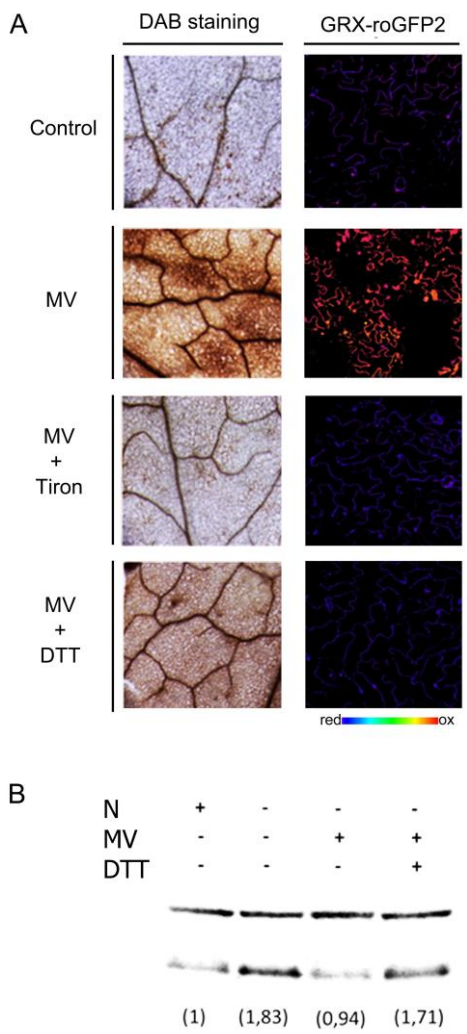
910
911 **Supplementary Figure 3**



Supplementary Figure 3: Effects of DTT and tiron on ROS production, cytoplasmic oxidation of the GSH:GSSG sensor probe (GRX-roGFP2), and autophagic flux in Arabidopsis plants exposed to 1 µM MV. (A) Production of H_2O_2 revealed by *in situ* staining with 3′,3′-diaininobenzidine (DAB staining) and cytoplasmic redox state (GRX-roGFP2). GRX-roGFP column is a ratiometric image. For pseudocolor display, the ratio was coded on a spectral color scale ranging from blue (fully reduced) to red (fully oxidized). Representative images are shown. At least 3 plants were analyzed. (B) Autophagic flux evaluated through the degradation of the GFP-ATG8a fusion protein by western blot. Seven-day old 35S:GFP-ATG8a expressing plants were exposed to the different treatments. Total protein (40 µg) was subjected to immunoblot analysis with anti-GFP antibodies. The GFP-ATG8a fusion has a predicted molecular weight of approximately 40 kDa (closed arrowheads); free GFP has a predicted molecular weight of 27 kDa (open arrowheads). Representative images are shown. Ratio: the numbers in brackets denote the GFP/GFP-ATG8 ratio relativized to the control condition. N: nitrogen; MV: methyl viologen; DTT: dithiothreitol.

Supplementary Figure 4

Sequence alignment of ATG4 proteases from different organisms
Colored by: identity

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1 ScATG4 ----------MQRWLQLWKMDLVQKVSH--G
2 HsATG4A isoform a ----------MESVLSKYEDQIT
3 HsATG4B ----------MDAATLTYD--T
4 CrATG4 ----------MSQALSLRKLSE--L
5 AtATG4A MKALCDRFVPQQCSSSSKSDTHD---KSPLVSDSGPSDNKSKFTLWSNVFTSSSSVSQPYRESSTSGHKQVCTTRNGWTAFVKRVSMASGAIRRFQE--R
6 AtATG4B MKAICDRFVPSKCSSSSTSEKRDISSPTSLVSDSASSDNKSNLTLCSDVVASSSPVSQLCREASTSGHNPVCTTHSSWTVILKTASMASGAIRRFQD--R
7 MpATG4 ----------MTTNSEQEEQATKLASNFWSKKVSEVSRSLHLKAGESLILGLMEKPRNQVRRLHD--K
8 PpATG4A ----------MRRLQE--L
9 PpATG4B ----------MQRLQE--L

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1 ScATG4 VFEGSSEEPAALMNHDYIVLGEVYPERDEESGAEQCEQDCRYRGEAVSDGFLSSLFGREISSYTKEFLLDVQSRVNFTYRTRFVPIARAPDGPSPLSLNL
2 HsATG4A isoform a IFTDYLEEYPDTD-ELVWILGKQHLLKTEKS----------KLLSDISARLWFTYRRKFSPIG----------
3 HsATG4B LRFAEFEDFPETS-EPVWILGRKYSIFTEKD----------EILSDVASRLWFTYRKNFPAIG----------
4 CrATG4 MHG-----TTSLSQHTICLLGKSYGDAGTGDQSKALDAEA----------LEAMLGDFRSRMWCTYRKDFPALG----------
5 AtATG4A VLGPNRTGLPSTT-SDVWLLGVCYKISADENSGETDTGTV----------LAALQLDFSSKILMTYRKGFEPFR----------
6 AtATG4B VLGPSRTGISSST-SEIWLLGVCYKISEGESSEEADAGRV----------LAAFRQDFSSLILMTYRRGFEPIG----------
7 MpATG4 IWGAKWTSGTASN-STIWVLGASYEFQPETR-DEPVA----------EFLLDFSSRVWLTYRRGFEALG----------
8 PpATG4A LLGPRFTAANASSGSEIWVLGLCYKVSADPN-NETLSVQA----------FEEFISDFTSRIWITYRKGFECVG----------
9 PpATG4B LLRHRFTAADSSSGGEIWVLGICYKVSADAN-DEAVSAHA----------FEEFLNDFSSRIWITYRKGFESLG----------

                 201                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                
Active site
1 ScATG4 LVRTNPISTIEDYIANPDCFNTDIGWGCMIRTGQSLLGNALQILHLGRDFRVN-GNESLERESKFVNWFNDTPEA--PFSLHNFVSAGTELSDKRPGEWF
2 HsATG4A isoform a ----------GTGPSSDAGWGCMLRCGQMMLAQALICRHLGRDWSWEKQKEQPKEYQRILQCFLDRKDC--CYSIHQMAQMGVGEG-KSIGEWF
3 HsATG4B ----------GTGPTSDTGWGCMLRCGQMIFAQALVCRHLGRDWRWTQRKRQPDSYFSVLNAFIDRKDS--YYSIHQIAQMGVGEG-KSIGQWY
4 CrATG4 ----------PSLLTSDVGWGCTLRSGQMLLAEAMMRVALGRDWQR--CSDNLEAVRPVVAALLDCAEA--PLSIHRICDAGGPAG-IVPGRWL
5 AtATG4A ----------DTTYTSDVNWGCMIRSSQMLFAQALLFHRLGRAWTKK-SELPEQEYLETLEPFGDSEPS--AFSIHNLIIAGASYG-LAAGSWV
6 AtATG4B ----------DTTYTSDVNWGCMLRSGQMLFAQALLFQRLGRSWRKKDSEPADEKYLEILELFGDTEAS--AFSIHNLILAGESYG-LAAGSWV
7 MpATG4 ----------QSKFTSDVGWGCMLRSGQMLLAQALVCHHLGRGWRLDPDQPLERKYLEILQSFGDCRGALYPFSIHNLLERGSPYG-LVAGSWL
8 PpATG4A ----------QSKLTSDVGWGCMLRSGQMLLAQALVCHYLGRSWRREPGQPCSQAYLQILQTFGDSESC--PFSIHNLLEAGHPFG-LAAGSWL
9 PpATG4B ----------ESKLTSDVGWGCMLRSGQILLAQALVCHYLGRTWRRNACQECLQEYLQILQSFGDSESC--SFSIHNLLEAGRPFG-LAAGSWL

                 301                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                
1 ScATG4 GPAATARSIQSL----------
2 HsATG4A isoform a GPNTVAQVLKKL----------
3 HsATG4B GPNTVAQVLKKL----------
4 CrATG4 GPWMLCKGLEALFSQLAGAQQEPLGLRLHVACGAGGGAPELHMDLIRAAMAPAAQSAATAGTGKAGTGKGSGAGADEGTGDKVGLEAAAGCSTGSAGPLR
5 AtATG4A GPYAICRAWESL----------
6 AtATG4B GPYAVCRSWESL----------
7 MpATG4 GPYALCRTIEAL----------
8 PpATG4A GPYALCRTLEAL----------
9 PpATG4B GPYALCRTLEAL----------

                 401                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                
1 ScATG4 ----------IYGFPE----------
2 HsATG4A isoform a ----------ALFDE----------WNSLAVYVSMD----------NTV
3 HsATG4B ----------AVFDT----------WSSLAVHIAMD----------NTV
4 CrATG4 CERSRSQVEEDVGVACKQPEGCGCAKQEVPVRGLPADLGLEAHDCGHPGHTGTGGDRQVTASGPGGPDDAGQSSAQGHGDREAVAGSRLQGPQRTEGTVA
5 AtATG4A ----------ACKKRKQ---TDSK--NQTLPMAV----------HIV
6 AtATG4B ----------ARKNKEE---TDDK--HKSFSMAV----------HIV
7 MpATG4 ----------ALADIEA---SKNEKQKRALPMAV----------CVV
8 PpATG4A ----------ARADREQ---SQKKGGKRALPFAV----------YVV
9 PpATG4B ----------AKADEDQ---NAKKGGKRALPFAV----------YVV

                 501                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                
1 ScATG4 --------CGIDD----------CIVSVSSGDIYENEVEKVFAE-------NPNSRILFLLGVKLGINAVNESYR
2 HsATG4A isoform a VIEDIKKMCRVLPL----------SADTAGDRPPDSLTASNQSKGTSAYC--SAWKPLLLIVPLRLGINQINPVYV
3 HsATG4B VMEEIRRLCRTSVP----------CAGATAFPADSDRHCNGFPAGAEVTNRPSPWRPLVLLIPLRLGLTDINEAYV
4 CrATG4 AGQFCSEPCGSPTVADAAVAAPGSAGREIASDTAPEQESASSSTAASSCCGEAEDAAAHGRRCRGVAAGTC-----GRGHSLLLLVPLTLGMDKINPVYI
5 AtATG4A SGSEDGERGGAPIL----------CIEDATK------SCLEFSKGQ------SEWTPIILLVPLVLGLDSVNPRYI
6 AtATG4B SGSEDGERGGAPIL----------CIEDVTK------TCLEFSEGE------TEWPPILLLVPLVLGLDRVNPRYI
7 MpATG4 SGDAEGERGGAPVL----------FIEDVTD------KCAEWEDGP------GKWAPLLVLVPLVLGLEKVNPRYL
8 PpATG4A SGEAEGERGGAPVL----------CVEDVAT------LCSKWREPT------EEWTPLLVLVPLVLGLDKVNPRYL
9 PpATG4B SGETEGDRGGAPVR----------CVEDAAV------LCSKWGEAT------EEWSPLVVLVPLVLGLDKLNPRYL

                 601                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                
1 ScATG4 ESICGILSSTQSVGIAGGRPSSSLYFFGYQGNEFLHFDPHIPQPAV---EDSFVE----------
2 HsATG4A isoform a DAFKECFKMPQSLGALGGKPNNAYYFIGFLGDELIFLDPHTTQTFVDTEENGTVNDQ----------
3 HsATG4B ETLKHCFMMPQSLGVIGGKPNSAHYFIGYVGEELIYLDPHTTQPAVEPTDGCFIPDE----------
4 CrATG4 PQLQQVLSWPQSVGIVGGRPSASLYVCGVQDASFIYLDPHEAQLAVRTPAATAAATAAAAVAAPAGGAAATVRAAAGGGGAGAGGQGGGATAWPPTGWLA
5 AtATG4A PSLVATFTFPQSVGILGGKPGASTYIVGVQEDKGFYLDPHEVQQVVTVNKETPDVDTS----------
6 AtATG4B PSLIATFTFPQSLGILGGKPGASTYIVGVQEDKGFYLDPHDVQQVVTVKKENQDVDTS----------
7 MpATG4 PSLRATFSFPQSLGIAGGKPGASHYLVGIQDDQALYLDPHETQQAVSISPDDVEADTH----------
8 PpATG4A PSLRATFTFPQSLGIAGGKPGASTYLIGVQDEQAMYLDPHENQQVVPVTPENLELDTS----------
9 PpATG4B PSLRATFTLPQSLGVAGGKPGASTHLIGVQGDQAMYLDPHENQQVFAVTPENLELDTS----------

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1 ScATG4 -----SCHT-SKFGKLQLSEMDPSMLIGILIKGEKDWQQWKLEVAESAIINVLAKRMDDFDVSCSMDDVESVSSNSMKKDASNNENLGVLEGDYVDIGAI
2 HsATG4A isoform a -----TFHCLQSPQRMNILNLDPSVALGFFCKEEKDFDNWCSLVQK-------EILKENLRMFELVQKHPSHWPPFVPPAKPEVTTTGAEFIDSTEQLEE
3 HsATG4B -----SFHCQHPPCRMSIAELDPSIAVGFFCKTEDDFNDWCQQVKK------LSLLGGALPMFELVELQPSHL------AQPDVLNLSLDSSDVERLERF
4 CrATG4 APQLGTYFC-DVVRVLPSAQLDPSLAIGFVCTSSAELEDLFARLQA------LATQHSSAPLMTLTTGS----------GAAVGC--GSDADFTDDVLEG
5 AtATG4A -----SYHC-NVLRYVPLESLDPSLALGFYCRDKDDFDDFCLRALK------LAEESNGAPLFTVTQTH----------TAINQSNYGFADDDSED----
6 AtATG4B -----SYHC-NTLRYVPLESLDPSLALGFYCQHKDDFDDFCIRATK------LAGDSNGAPLFTVTQSH----------RRNDCGI-AETSSSTETSTE
7 MpATG4 -----SYHC-REVRRMPLDAIDPSLALGFYCKSREDFDDLCTRASE------LATQSQGAPMFTVAQGRSRKHST--DGFNLDKLSIDVQESSTKDHVNA
8 PpATG4A -----SYHC-STVRRLPLDTIDPSLAIGFYCRDRAEFDDLCARSSE------LAKQSNGAPMFTVAEKSA-------PPKPADSCNLDLEESIPKDP---
9 PpATG4B -----FYHC-SVVRRLPLDSIDPSLAIGFYCRDRAEFDDLCARSSE------LVKQYNGAPIFTVAEKSA-------PSKLQNSSDMGLEESFSKDP---

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1 ScATG4 FPHTTNTEDVDEYDCFQDIHCKKQKIVVMGNTHTVNANLTDYEVEGVLVEKETVGIHSPIDEKC
2 HsATG4A isoform a F---DLEED---FEILSV----------
3 HsATG4B FD--SEDED---FEILSL----------
4 CrATG4 GTGQQQLDE---WELV----------
5 AtATG4A ----EREDD---WQML----------
6 AtATG4B ISGEEHEDD---WQLL----------
7 MpATG4 DTASSPEDD---WQIL----------
8 PpATG4A ----SSEDD---WQML----------
9 PpATG4B ----SSEDD---WQML----------
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958 **Supplementary Figure 4:** Sequence alignment of ATG4 proteases from different organisms
959 including *Saccharomyces cereviseae* (QHB11163.1), *Homo sapiens* (ATG4A- NP_001308219.1,
960 ATG4B, NP_847896.1) *Chlamydomonas reinhardtii* (Cre12.g510100.t1.1), *Arabidopsis thaliana*
961 (ATG4A- AT2G44140.1, ATG4B- AT3G59950.1), *Marchantia polymorpha* (Mapoly0153s0038.1.p)
962 and *Physcomitrium patens* (ATG4A- Pp3c11_22820V3.1.p, ATG4B, Pp3c7_280V3.1.p). In orange
963 is labeled the conserved active site (WGCT/ML/IR) of these proteases including the catalytic
964 Cys77 (number according to HsATG4A) that is essential for ATG4 activity (Tanida et al., 2004).
965 Note that HsATG4A and HsATG4B contain two conserved cysteine residues Cys81 and Cys92, or
966 Cys78 and Cys89 (numbers according to HsATG4A or HsATG4B, respectively, black arrows), but
967 these cysteine residues are not conserved in yeasts or other eukaryotes as algae or plant ATG4s.
968 H_2O_2, at the site of Cys81/Cys78, is an oxidative signal that inactivates the delipidation activity
969 of ATG4 at the site of autophagosome formation promoting lipidation of ATG8. In the case of
970 HsATG4B, Cys292 and Cys361 (Cys labeled in red) which are not conserved in other species, are
971 regulated by redox signals to influence ATG4B activity and autophagy. In yeast ATG4, its activity
972 is redox regulated by oxidoreduction of a single disulfide bond between Cys-338 and Cys-394
973 (Cys labeled in blue). In Chlamydomonas, Cys400 is identified as the key site participating in Atg4
974 redox regulation (Cys labeled in green).
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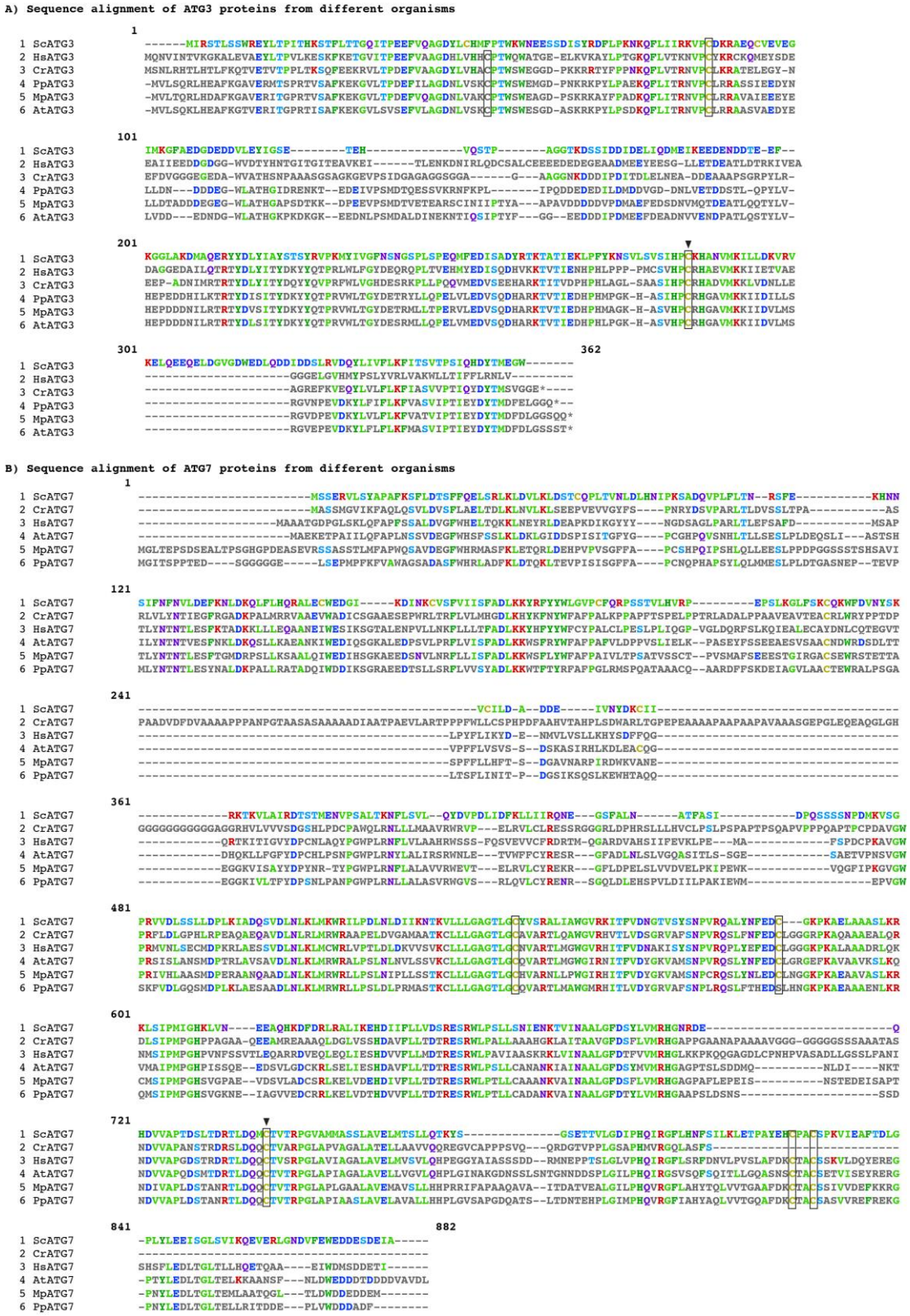
Supplementary Figure 5



1005 **Supplementary Figure 5:** (A) Sequence alignment of ATG3 proteins from different organisms
1006 including *Saccharomyces cerevisiae* (QHB11385.1), *Homo sapiens* (NP_001265641.1),
1007 *Chlamydomonas reinhardtii* (Cre02.g102350_4532.1), *Arabidopsis thaliana* (AT5G61500.1),
1008 *Marchantia polymorpha* (Mapoly0003s0208.1.p) and *Physcomitrium patens*
1009 (Pp3c8_11900V3.1.p). The catalytic Cys264 (number according to HsATG3) is conserved in all
1010 proteins studied (black box with a black arrow). (B) Sequence alignment of ATG7 proteins from
1011 different organisms including *Saccharomyces cerevisiae* (QHB09162.1), *Homo sapiens*
1012 (NP_001336161.1), *Chlamydomonas reinhardtii* (Cre03.g165215.t1.1), *Arabidopsis thaliana*
1013 (AT5G45900.1), *Marchantia polymorpha* (Mapoly0015s0071.1.p) and *Physcomitrium patens*
1014 (Pp3c24_8100V3.1.p). The catalytic Cys572 (number according to HsATG7) is conserved in all
1015 proteins studied (black box with a black arrow). Other potential cysteine residues which are
1016 conserved may serve as potential candidates for further investigation. Under nonreducing
1017 conditions, when ATG3 and ATG7 enzymes are inactive, form stable thioester complexes with
1018 LC3 (ATG8). Under pro-oxidizing conditions, loss in this stable interaction with LC3 during active
1019 autophagy sensitizes ATG3 and ATG7 to thiol oxidation and leads to the formation of an
1020 intermolecular disulfide-bound complex between the Cys264 from ATG3 and the Cys572 from
1021 ATG7, which inhibit ATG8 lipidation (Frudd et al., 2018). Other cysteine residues which are
1022 conserved (black boxes) are potential candidates for further studies.
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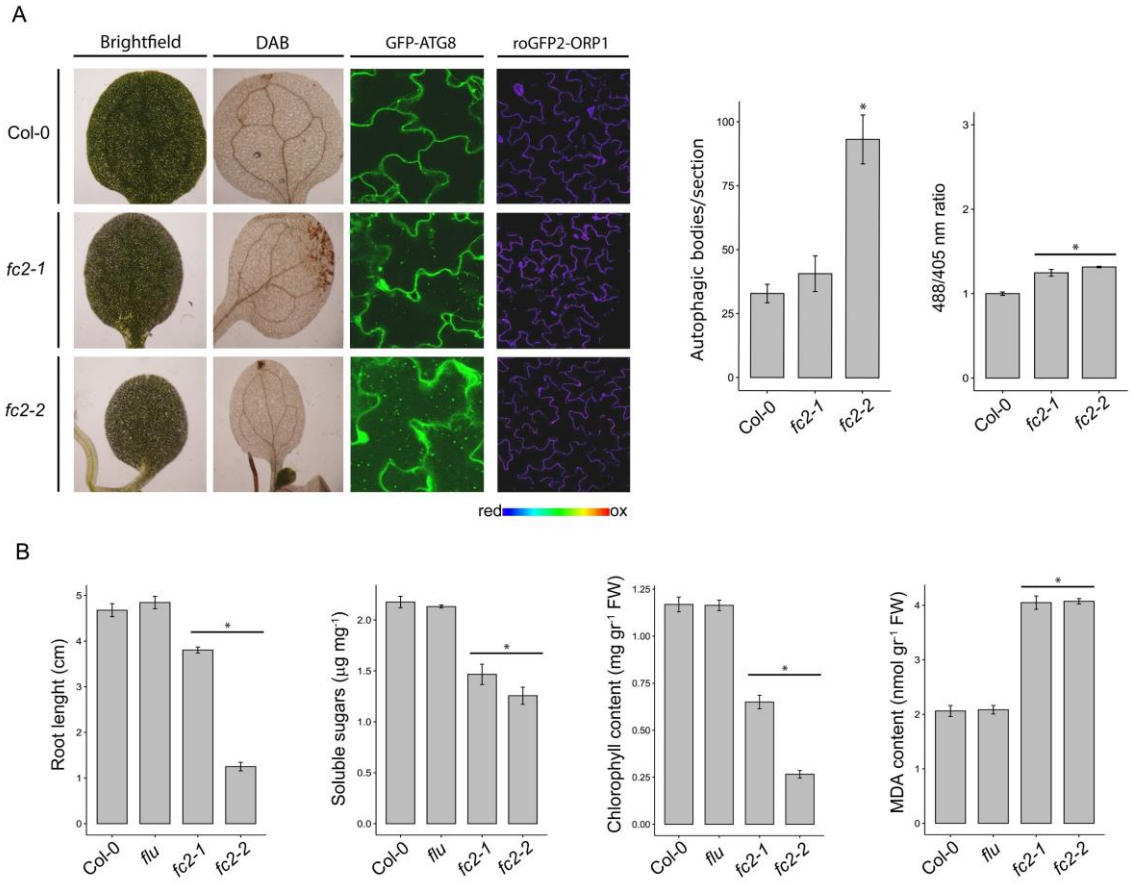
1050 **Supplementary Figure 6**



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1052 **Supplementary Figure 6:** Characterization of *fc2* and *flu* mutant plants under continuous light
1053 conditions (control conditions). Wild type (Col-0), *fc2* and *flu* mutant plants were grown 7 days
1054 under continuous light conditions and different growth and physiological parameters were
1055 evaluated. (A) Production of H_2O_2 was revealed by *in situ* staining with 3′,3′-diaininobenzidine
1056 (DAB); autophagic bodies accumulation (GFP-ATG8 columns) and the cytoplasmic redox state
1057 (GFP2-Orp1 column) analyzed by confocal microscopy. To induced autophagic bodies
1058 accumulation (GFP-ATG8 column), MS liquid medium was supplemented with 1 µM
1059 Concanamycin A. GFP2-Orp1 column is a ratiometric image. For pseudocolor display, the ratio
1060 was coded on a spectral color scale ranging from blue (fully reduced) to red (fully oxidized).
1061 Representative images are shown. At least 4 plants were analyzed. Bar plots show the average
1062 number of autophagic bodies and the average of cytoplasmic redox state. The average of
1063 autophagic bodies number was calculated from single-plane confocal images. Two sections from
1064 at least 4 plants were analyzed. The average of cytoplasmic redox state is expressed as the ratio
1065 of the fluorescence intensities measured at 488 and 405 (data expressed relative to Col-0). At
1066 least 4 plants were analyzed. (B) Average root length, soluble sugar, chlorophyll and MDA
1067 content in the different genotypes after growing seven days under continuous light conditions
1068 (*$P < 0.05$; error bars: means ± SEM, DGC test).
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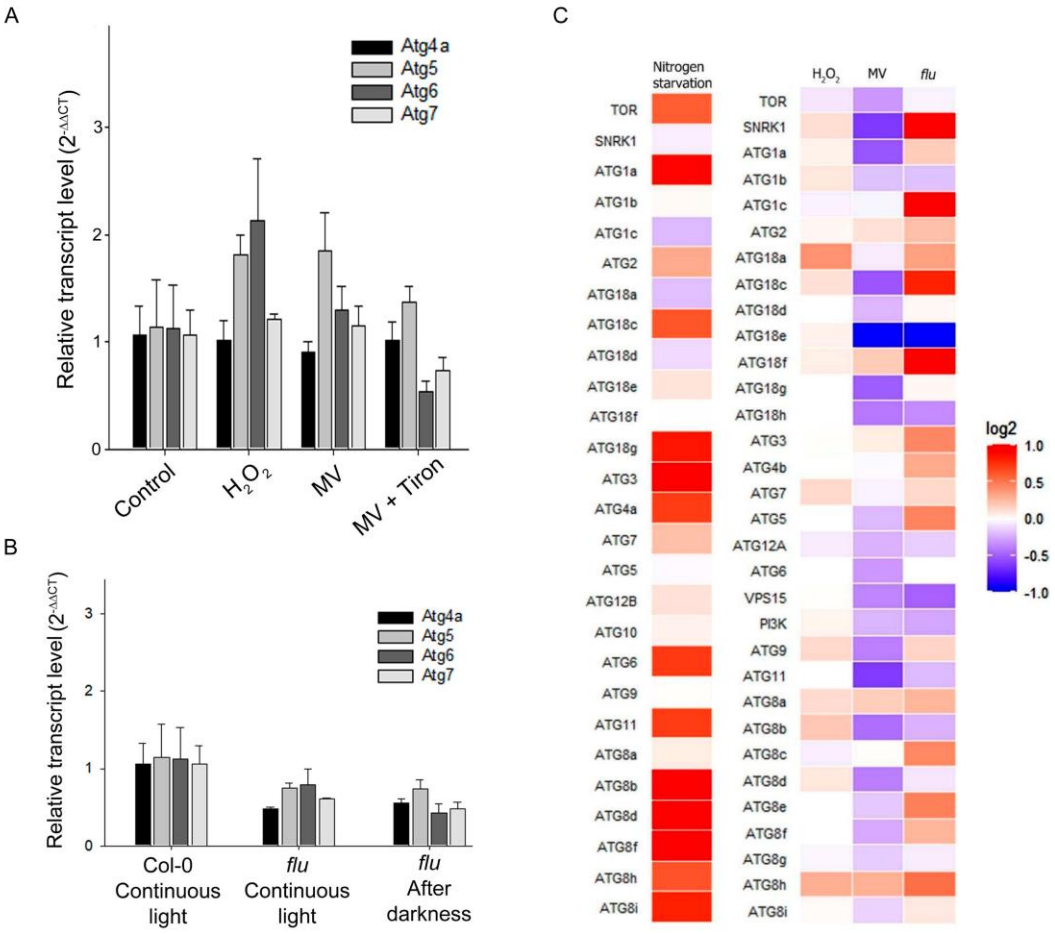
1071 **Supplementary Figure 7**



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1073 **Supplementary Figure 7:** Transcript level of *Atg* genes in Arabidopsis plants subjected to
1074 different oxidative treatments. (A) Seven-day old wild type Arabidopsis plants (Col-0) were
1075 exposed to oxidative stress conditions for 4 h (10 mM H_2O_2, 1 µM methyl viologen (MV), 1 µM
1076 methyl viologen + 10 mM tiron (MV + Tiron)), followed by the analysis of the transcript levels of
1077 *Atg* genes through RT-qPCR. Data are relative to control condition (assigned a value of 1). (B)
1078 Seven-day old *flu* mutant plants grown under continuous light conditions were exposed to
1079 darkness for 8 h and re-exposed to light for 8 h or kept in continuous light (control), and the
1080 expression of *Atg* genes were analyzed by RT-qPCR. Data are relative to Col-0 genotype (assigned
1081 a value of 1). (C) Publicly available microarray data were utilized to compare the expression of
1082 *Atg* genes under various conditions, including nitrogen deficiency (Rubin *et al.*, 2009), treatment
1083 with 10 mM H_2O_2 for 24 h (Willems *et al.*, 2016), treatment with 10 µM MV for 2 h (Scarpeci *et*
1084 *al.*, 2008), and the examination of *flu* mutant plants subjected to an 8-hour cycle of darkness
1085 followed by 2 h of light (Op Den Camp *et al.*, 2003). Expression values were normalized to the
1086 corresponding control conditions employed in each study and were expressed as $\log_2$ values.
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