Regulation of Chlorophagy during Photoinhibition and Senescence: Lessons from Mitophagy

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Title: Regulation of Chlorophagy during Photoinhibition and Senescence: Lessons from Mitophagy

Running head: Regulation of Chlorophagy

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Abbreviations: APX, ascorbate peroxidase; *ATG*, *AUTOPHAGY*; ATI, Autophagy 8-interacting protein; δTIP, delta tonoplast intrinsic protein; DIC; differential interference contrast; FC2, ferrochelatase 2; FUNDC1, FUN14 domain containing 1; GFP, green fluorescent protein; HL, high visible light; NIX, Nip3-like protein X; NYC1, Non-yellow coloring 1; PGR5, proton gradient regulation 5; PI3K, phosphatidylinositol 3-kinase; PINK1, PTEN-induced putative kinase 1; PPFDs, photosynthetic photon flux density; PS, photosystem; PUB4, Plant U-Box 4; RBCS, Rubisco small subunit; RFP, red fluorescent protein; ROS, reactive oxygen species; SOD, superoxide dismutase; SP1, suppressor of plastid protein import 1;TEM, transmission electroscopic microscopy; TOC, translocon on the outer chloroplast membrane; TOM, translocase of outer membrane; UV, ultraviolet; VIPP1, Vesicle-inducing protein in plastids 1

Abstract

Light energy is essential for photosynthetic energy production and plant growth. Chloroplasts in green tissues convert energy from sunlight into chemical energy via the electron transport chain. When the level of light energy exceeds the capacity of the photosynthetic apparatus, chloroplasts undergo a process known as photoinhibition. Since photoinhibition leads to the overaccumulation of reactive oxygen species (ROS) and the spreading of cell death, plants have developed multiple systems to protect chloroplasts from strong light. Recent studies have shown that autophagy, a system that functions in eukaryotes for the intracellular degradation of cytoplasmic components, participates in the removal of damaged chloroplasts. Previous findings also demonstrated an important role for autophagy in chloroplast turnover during leaf senescence. In this review, we describe the turnover of whole chloroplasts, which occurs via a type of autophagy termed chlorophagy. We discuss a possible regulatory mechanism for the induction of chlorophagy based on current knowledge of photoinhibition, leaf senescence, and mitophagy – the autophagic turnover of mitochondria in yeast and mammals.

Keywords: autophagy, chlorophagy, chloroplasts, photoinhibition, mitophagy, senescence

Introduction

Plants absorb light energy from the sun using chlorophyll pigments and convert the energy from visible light (wavelengths of 400 to 700 nm) into chemical energy via the photosynthetic electron transport chain, comprising photosystem II (PSII), the cytochrome *b6f* complex, photosystem I (PSI) and the ATP synthase complex. These photosynthetic reactions occur in the chloroplast. The conversion of light energy can potentially damage the photosynthetic machinery via a process known as photoinhibition (Aro et al. 1993; Li et al. 2009). Plants concomitantly absorb ultraviolet (UV)-A (wavelengths of 315 to 400 nm) and UV-B (280 to 315 nm) radiation, which can directly damage macromolecules in the cell, such as proteins, DNA and lipids (Takahashi and Badger 2011; Kataria et al. 2014). UV-related damage may enhance photoinhibition (Takahashi and Badger 2011). ROS are actively produced during photoinhibition and directly cause further oxidative damage to chloroplasts (Asada 2006). Consequently, plants have developed diverse chloroplast protection systems to quench excess light energy, repair photodamaged proteins and scavenge ROS (Takahashi and Badger 2011); however, the fate of photodamaged, collapsed chloroplasts is not clearly understood.

Autophagy: a major intracellular degradation system for cytoplasmic components in eukaryotes

Organelle turnover in eukaryotic cells is widely achieved via autophagy-related transport into lytic organelles, including lysosomes in animal cells and the vacuole in yeast and plant cells (Ohsumi 2001). Macroautophagy is a well-characterized

88 autophagic process by which cytoplasmic components are engulfed by double-
89 membrane-bound vesicles known as autophagosomes. The outer membrane of the
90 autophagosome then fuses with the lysosomal or vacuolar membrane and releases the
91 inner membrane-bound autophagic body into the lysosomal or vacuolar lumen
92 (Nakatogawa et al. 2009; Mizushima and Komatsu 2011). During another type of
93 autophagy termed microautophagy, cytoplasmic components are directly engulfed by
94 the invaginated membranes of the lysosome or vacuole, and the sequestered material is
95 subsequently degraded (Li et al. 2012). This process is well characterized in the
96 methylotrophic yeast *Pichia pastoris* (Oku and Sakai 2016), in which the switch from
97 the use of methanol to glucose as the cell's energy source activates the microautophagic
98 digestion of peroxisomes.
99 *AUTOPHAGY* (*ATG*) genes were originally identified in the budding yeast
100 *Saccharomyces cerevisiae* (Tsukada and Ohsumi 1993). To date, 41 *ATGs* have been
101 identified in yeast, including 15 (*ATG1–10*, *12–14*, *16*, *18*) "core" *ATGs* that are
102 required for all types of autophagy (Nakatogawa et al. 2009). Core ATGs are classified
103 into four subgroups: 1) ATG1 and ATG13 are components of the ATG1 kinase complex,
104 2) ATG6 and ATG14 are components of the autophagy-specific phosphatidylinositol 3-
105 kinase (PI3K) complex, 3) ATG2 and ATG18 form a complex with membrane-anchored
106 ATG9 and 4) the remaining core ATGs participate in the two ubiquitin-like conjugation
107 systems that facilitate ATG8 lipidation and autophagosomal membrane elongation
108 (Nakatogawa et al. 2009). Through the two ubiquitin-like cascades, ATG8 is conjugated
109 with a lipid, phosphatidylethanolamine, subsequently forming the autophagosomal
110 membrane (Ichimura et al. 2000). These core autophagy components are mainly

111 involved in autophagosome formation, and their orthologs have been identified in
112 various plant species (Meijer et al. 2007; Chung et al. 2009; Zhou et al. 2015).
113 Autophagy mediates the bulk digestion of cytoplasmic components and facilitates
114 the recycling of released molecules, such as amino acids, especially under starvation
115 conditions. In addition, specific organelles or proteins are selectively transported into
116 lytic organelles as selective cargoes of autophagosomes under various conditions
117 (Anding and Baehrecke 2017). This selective autophagy process leads to the removal of
118 dysfunctional organelles; for example, dysfunctional mitochondria are removed through
119 a selective autophagy process termed mitophagy in yeast and mammals (Youle and
120 Narendra 2011; Kanki et al. 2015).

121 **Chlorophagy removes whole photodamaged chloroplasts**

122 Studies on *Arabidopsis thaliana* mutants of core *ATGs* indicate that the core autophagy
123 machinery for the initiation and elongation of the autophagosomal membrane has been
124 conserved in plants (Li and Vierstra 2012; Liu et al. 2012b; Yoshimoto 2012). The
125 establishment of *in vivo* monitoring methods for plant autophagy based on fluorescent
126 marker proteins of the autophagosomal membrane or organelles has further facilitated
127 studies of the involvement of autophagy in the intracellular turnover of plant organelles
128 (Yoshimoto et al. 2004; Thompson et al. 2005). A recent study investigated the
129 possibility that autophagy participates in the turnover of photodamaged chloroplasts
130 under stress conditions (Izumi et al. 2017). This study revealed that whole chloroplasts
131 are transported into the vacuole following photodamage caused by exposure to strong
132 visible light or UV-B through an autophagic process termed chlorophagy. This

phenomenon was observed in true rosette leaves of Arabidopsis plants grown in soil under a 12 h-light/12 h-dark photoperiod using fluorescent lamps (140 μmol m^{-2} s^{-1}) at 23°C. When plants grown under these conditions were exposed to strong visible light of various photosynthetic photon flux densities (PPFDs; 800, 1200, 1600, 2000 μmol m^{-2} s^{-1}) for 3 h, chlorophagy was only observed after exposure to more than 1,200 μmol m^{-2} s^{-1} PPFD (Izumi et al. 2017). Natural sunlight includes visible light, UV-A and UV-B. Exposure of chamber-grown Arabidopsis plants to natural sunlight also induces chlorophagy (Izumi et al. 2017), through sunlight damage.

Methods for assessing chlorophagic activity

Figure 1 shows the current methods used to detect and assess chlorophagic activity in Arabidopsis. When transgenic plants expressing stroma-targeted green or red fluorescent protein (GFP or RFP) are grown under normal conditions without photodamage treatment, all chloroplasts exhibiting chlorophyll autofluorescence produce signals from stroma-targeted fluorescent protein when observed under a confocal microscope (Izumi et al. 2017; Fig. 1A). At 2 d after a 2 h exposure to high levels of visible light (HL; 2,000 μmol m^{-2} s^{-1}), chloroplasts lacking stroma-targeted fluorescent protein signals that appear to move randomly are observed in the central regions of mesophyll cells (Fig. 1A, arrowheads), specifically in the central vacuole, as chloroplasts lacking stroma-targeted RFP were observed inside the tonoplast (labeled by GFP; Fig. 1B, arrowheads). Transmission electron microscopy (TEM) also revealed that chloroplasts accumulate in the vacuole after HL exposure (Fig. 1C, arrowheads). These chloroplasts have retained their thylakoid membranes but have lost their stromal

components, which is consistent with confocal microcopy observations of vacuolar chloroplasts labeled with fluorescent protein markers. It is thought that when chloroplasts are incorporated into the vacuole via chlorophagy, envelope and stromal components are degraded and diffuse before the thylakoid structures, including chlorophyll, are digested; such chloroplasts appear as stromal-marker-deficient chloroplasts under confocal microscopy (Fig. 1, arrowheads). TEM images show that vacuolar chloroplasts are partially fragmented, supporting the notion that vacuolar chloroplasts are in the process of being digested (Fig. 1C). Such observations led to the discovery of chlorophagy, a process by which whole photodamaged chloroplasts are transported into the central vacuole (Fig. 1D; Izumi et al. 2017).

Fluorescently labeled stroma-targeted proteins can be used to easily distinguish vacuolar chloroplasts (resulting from chlorophagy) from cytoplasmic chloroplasts (Fig. 1). The direct observation and counting of vacuole-incorporated chloroplasts using plants expressing stroma-targeted fluorescent proteins is a simple, reliable method for assessing chlorophagic activity. In fact, the number of stroma-deficient vacuolar chloroplasts increases in response to greater chloroplast damage, as represented by the larger decline in the maximum quantum yield of PSII (*Fv*/*Fm*; Izumi et al. 2017).

Studies examining organelle-targeted autophagy frequently involve biochemical assays using organelle marker proteins fused with fluorescent proteins, in which free fluorescent proteins derived from vacuolar degradation of the fusion proteins are detected by immunoblot analysis of protein extracts (Mizushima et al. 2010). For instance, mitophagic activity in yeast has been assessed by detecting free GFP released via the vacuolar degradation of the mitochondria-targeted fusion protein Om45-GFP

178 (consisting of the C-terminus of the mitochondrial outer membrane protein Om45 and
179 GFP; Kanki et al. 2009). The establishment of similar biochemical methods to
180 specifically monitor the occurrence of chlorophagy in combination with other
181 techniques might allow for the future quantitative evaluation of chlorophagy induction
182 under various conditions.

183 **The relationship between photoinhibition and chlorophagy**

184 During PTEN-induced putative kinase 1 (PINK1) and Parkin (PINK1/Parkin)-mediated
185 mitophagy in mammals (Fig.1B), depolarized mitochondria that lose transmembrane
186 potential ($\Delta\Psi$) across the inner envelope for ATP synthesis become the targets of
187 selective removal (Youle and Narendra 2011). Similarly, damaged chloroplasts suffering
188 from a specific damage might be selectively removed in individual mesophyll cells
189 during chlorophagy. The decline in *Fv/Fm* represents the extent of photoinhibition, and
190 chlorophagy is activated in response to larger declines in *Fv/Fm* (Izumi et al. 2017);
191 therefore, we postulate that photoinhibition-associated chloroplast damage is closely
192 related to the selective recognition of the cargo of chlorophagy.
193 Multiple systems prevent the occurrence of photoinhibition in chloroplasts.
194 Excessive light energy absorbed by the PSII light-harvesting complex is quenched as
195 heat energy through a mechanism known as thermal energy dissipation (Havaux and
196 Niyogi 1999). The efficiency of this energy dissipation corresponds to the extent of ΔpH
197 across the thylakoid membrane (Jahns and Holzwarth 2012). Cyclic electron flow
198 around PSI can produce high ΔpH levels during photosynthesis (Shikanai and
199 Yamamoto 2017). Metabolic processes across chloroplasts, mitochondria and

200 peroxisomes, such as the malate–oxaloacetate shuttle and photorespiration, likely help
201 dissipate excessive reducing power (Yamori 2016). When the reducing power produced
202 by excess light energy is not sufficiently dissipated, the photosystems produce ROS,
203 including singlet oxygen ($^1O_2$) from PSII or hydrogen peroxide (H_2O_2) and superoxide
204 (O_2^-) from PSI (Asada 2006). Chloroplasts have scavenging systems for ROS: $^1O_2$ is
205 detoxified by carotenoids that closely localize around the PSII reaction centers (Ramel
206 et al. 2012), O_2^- is quickly dismutated to H_2O_2 by superoxide dismutase (SOD), and
207 H_2O_2 is detoxified by ascorbate peroxidase (APX; Asada 2006). Accumulated ROS and
208 increasing reducing power primarily damage the D1 reaction center within PSII (Aro et
209 al. 1993). Damaged D1 turns over very rapidly via the cooperative activity of two types
210 of intrachloroplastic proteases, FtsH and Deg, and is replaced by newly synthesized D1
211 (Kato et al. 2012). Photoinhibition appears when light energy exceeds the capacity of
212 these protection and repair mechanisms. Such conditions are sometimes caused by the
213 interference of additional abiotic stresses, such as drought and low temperatures, with
214 photosynthetic reactions (Yamori 2016). Even under normal light conditions that do not
215 induce strong photoinhibition (100 μmol m^{-2} s^{-1}), mutants of a major subunit of FtsH
216 (FtsH2) showed compromised D1 degradation and accumulated more ROS in their leaf
217 chloroplasts than in wild-type (Kato et al. 2009). Therefore, PSII damage constantly
218 occurs under normal (non-stressed) growth conditions, but photoinhibition of PSII does
219 not emerge when the PSII repair system sufficiently restores such damage.
220 It is thought that if a chloroplast sustains local damage that can be sufficiently
221 repaired by intrachloroplastic systems, and chloroplast functions can be maintained, the
222 chloroplast would be subjected to local repair systems instead of total degradation via

223 chlorophagy. Therefore, given that PSII damage occurs constantly and is rapidly
224 repaired by proteases, PSII photoinhibition is unlikely to be the direct trigger of
225 chlorophagy.

226 In contrast to PSII, PSI does not have a quick repair system; PSI repair is a
227 relatively slow process compared to that of PSII, requiring several days for completion
228 (Scheller and Haldrup 2005). PSI damage mainly involves the O_2^--induced damage of
229 iron-sulfur (FeS) clusters within the PSI reaction centers. PSI damage was originally
230 considered to occur only in response to specific treatments under experimental
231 conditions, such as exposure to moderate light with chilling treatment (Sonoike 1998);
232 conversely, recent studies have indicated that PSI damage may constantly occur under
233 fluctuating light conditions, such as in natural sunlight (Yamori 2016). PROTON
234 GRADIENT REGULATION5 (PGR5) is a PSI-associated protein that is required for
235 the generation of the ΔpH across the thylakoid membrane through the activation of
236 cyclic electron flow (DalCorso et al. 2008; Shikanai and Yamamoto 2017). The
237 Arabidopsis *pgr5* mutant accumulates more severe damage to PSI during HL
238 illumination compared to wild-type plants, and the growth of this mutant is strongly
239 suppressed under experimentally fluctuating light conditions, i.e., exposure to repeated
240 cycles of 5-min of moderate light and 1-min of strong light throughout the day (Suorsa
241 et al. 2012). Thus, the accumulation of PSI damage upon sudden irradiation under
242 fluctuating light conditions likely leads to fatal damage.

243 In the Arabidopsis chloroplast, stromal APX (sAPX) and thylakoid APX (tAPX)
244 help scavenge O_2^- and H_2O_2 (Maruta et al. 2012). The possible involvement of O_2^- and
245 H_2O_2 accumulation in the induction of chlorophagy was suggested by the observation

246 that UV-B damage-induced chlorophagy is activated in *tAPX* mutant plants compared to
247 wild type (Izumi et al. 2017). Therefore, O_2^--related damage, including PSI
248 photoinhibition, might be linked to the induction of chlorophagy.

249 **Photoinhibition may damage the envelope**

250 The core autophagy machinery is limited to the cytoplasm, and the envelope acts as a
251 border between the chloroplast and cytoplasm. During PINK1/Parkin-mediated
252 selective mitophagy in mammalian cells, the modification of the outer envelope is a key
253 induction signal for this process, which follows the loss of $\Delta\Psi$ across the inner
254 envelope. Therefore, it is possible that altered envelope integrity may act as a trigger for
255 the induction of chlorophagy. In support of this theory, recent studies have established
256 that the chloroplast envelope can accumulate damage and that VESICLE-INDUCING
257 PROTEIN IN PLASTIDS1 (VIPP1) plays an important role in maintaining envelope
258 integrity (Zhang et al. 2012). The VIPP1 homolog in *Escherichia coli,* Phage Shock
259 Protein A, helps maintain plasma membrane integrity. In plants, VIPP1 binds to the
260 membrane and functions in membrane remodeling (Heidrich et al. 2017). VIPP1-GFP
261 fusion protein localizes around the chloroplast envelope in the form of large particles
262 approximately 1 μm in diameter that appear to move quickly around chloroplasts during
263 osmotic stress (Zhang et al. 2012). VIPP1 has an intrinsically disordered region in its C-
264 terminus; deletion of the C-terminal region of VIPP1-GFP fusion protein led to
265 increased aggregation of these particles, thereby inhibiting their active movement and
266 preventing them from protecting the chloroplast membrane (Zhang et al. 2016b).
267 *VIPP1-GFP*-overexpressing Arabidopsis plants showed enhanced tolerance to heat

268 shock, but the expression of VIPP1 with a truncated C-terminus increased sensitivity to
269 this stress (Zhang et al. 2016b). These reports highlight the importance of protecting the
270 chloroplast membrane during plant stress responses.

271 *VIPP1*-knockdown Arabidopsis plants have abnormal, swollen chloroplasts,
272 indicating that the integrity of the chloroplast envelopes in these plants is impaired.
273 Swollen chloroplasts are also observed in seedlings of an Arabidopsis mutant of *NON-*
274 *YELLOW COLORING1* (*NYC1*), encoding an enzyme that degrades chlorophyll
275 (Nakajima et al. 2012); *nyc1* seedlings contain chlorotic cotyledons with swollen
276 chloroplasts (Zhang et al. 2016a). This phenomenon is likely caused by chlorophyll-
277 related photooxidative damage, since the number of seedlings with chlorotic cotyledons
278 increase with increasing PPFD during growth. Overexpressing *VIPP-GFP* in *nyc1*
279 plants restored their abnormal chloroplast shape and defective cotyledon phenotypes
280 (Zhang et al. 2016a). These results indicate that the envelope is a target of
281 photooxidative damage within chloroplasts and that VIPP1 can alleviate such envelope
282 damage.

283 In UV-B-damaged Arabidopsis leaves, few chloroplasts exhibit ruptured envelopes,
284 similar to those found in the cytoplasm of UV-B-damaged *atg* plants (Izumi et al. 2017).
285 TEM observations of mesophyll cells in UV-B-damaged *atg* leaves revealed normal as
286 well as abnormal chloroplasts with altered shapes and disorganized thylakoid
287 membranes. Treatment of tobacco leaf cells with methyl viologen, which enhances the
288 production of O_2^- within PSI, can lead to the rupture of the envelope (Kwon et al. 2013),
289 indicating that envelope can suffer ROS-mediated damage. As shown in Figure 1C,
290 some chloroplasts in HL-damaged mesophyll cells have abnormal shapes. In sum, the

291 extent of envelope damage and the related morphological changes to chloroplasts as a
292 result of ROS production around PSII and PSI during the induction of chlorophagy
293 should be a major focus of further study.

294 **Regulatory mechanisms of mitophagy to remove damaged mitochondria in yeast**
295 **and mammals**

296 The mitophagy regulatory mechanism for mitochondrial quality control has been
297 extensively studied in yeast and mammals. During PINK1/Parkin-mediated mitophagy
298 in mammals, depolarized mitochondria are eliminated, as mentioned previously. In
299 healthy mitochondria, PINK1 is imported into mitochondria and subsequently degraded
300 by the inner membrane-localized serine protease PARL (Jin et al. 2010). The $\Delta\Psi$ across
301 the inner membrane is also required for mitochondrial protein import; thus, its loss
302 allows PINK1 to accumulate on the TOM (translocase of the outer membrane) complex
303 (Matsuda et al. 2010; Narendra et al. 2010; Vives-Bauza et al. 2010; Lazarou et al.
304 2012). The accumulated PINK1 phosphorylates ubiquitin and the ubiquitin E3 ligase,
305 Parkin, to activate Parkin-mediated ubiquitination of mitochondria, thereby leading to
306 the build up of ubiquitin chains on mitochondrial outer membrane proteins (Koyano et
307 al. 2014). PINK1 and Parkin-mediated ubiquitination recruit various autophagic
308 receptors that bind to autophagosome-anchored LC3 (a mammalian homolog of ATG8;
309 Lazarou et al. 2015). These molecular events allow for the transport of depolarized
310 mitochondria as a specific cargo of autophagosomes. Therefore, PINK1 and Parkin-
311 mediated ubiquitination act as inducers, allowing dysfunctional mitochondria to be
312 selectively eliminated.

During mitophagy in yeast, ATG32 acts as an autophagic receptor that is directly anchored to the outer membranes of oxidized mitochondria and interacts with ATG8 (Kanki et al. 2009; Okamoto et al. 2009). ATG proteins with ATG8-interacting motifs also participate in the selective turnover of other organelles in yeast. For example, ATG39 and ATG40 were identified (in a co-immunoprecipitation assay of yeast ATG8) as the autophagic receptors of nucleus- or endoplasmic reticulum (ER)-targeted autophagy (nucleophagy or ER-phagy; Mochida et al. 2015).

The roles of plant ATG8-interacting proteins and chloroplast-associated ubiquitination in organelle turnover

To selectively remove collapsed chloroplasts via chlorophagy in plant cells, these chloroplasts must be recognized by a specific protein that functions in a manner similar to PINK1 and ATG32 during mitophagy in mammalian cells and yeast, respectively. Three AUTOPHAGY8-INTERACTING PROTEINS (ATIs) have been identified in plants. ATI1 and 2 interact with the ER or plastids, forming small vesicles during sugar starvation (Honig et al. 2012; Michaeli et al. 2014), and ATI3 may be involved in ER turnover during ER stress (Zhou et al. 2018). Thus, ATI1–3 are unlikely to be the autophagic receptors that trigger photodamage-induced chlorophagy.

A recent genetic screen indicated that the selective removal of chloroplasts involves ubiquitination (Woodson et al. 2015). When etiolated seedlings of the plastid-localized *FERROCHELATASE2* Arabidopsis mutant, *fc2*, are transferred from darkness to light, $^1O_2$ accumulates in their chloroplasts. This ROS accumulation causes the death of photosynthetic cells and impairs plant greening. A suppressor mutant of this inhibited

335 greening phenomenon has an additional single amino acid substitution in PLANT U-
336 BOX4 (PUB4), a cytosol-localized ubiquitin E3 ligase. In double mutants of *FC2* and
337 *PUB4*, the digestion of whole chloroplasts in the cytoplasm is suppressed compared to
338 *fc2* single mutants, even though $^1O_2$ accumulation is not affected in these mutants.
339 Therefore, PUB4-related ubiquitination triggers the degradation of $^1O_2$-accumulating
340 chloroplasts.

341 TEM images of greening *fc2* plants suggest that entire chloroplasts are digested in
342 the cytoplasm and that these digested chloroplasts interact with the central vacuole via
343 globule-like structures (Woodson et al. 2015). By contrast, during chlorophagy, whole
344 chloroplasts that have retained thylakoid membranes and exhibit chlorophyll
345 autofluorescence accumulate in the vacuolar lumen (Fig. 1). These distinct observations
346 suggest that PUB4-related ubiquitination is not a simple trigger of chlorophagy and that
347 it controls another pathway that specifically degrades $^1O_2$-accumulating chloroplasts.

348 In the cytoplasm, ubiquitinated proteins are generally degraded by the 26S
349 proteasome complex (Vierstra 2012). SUPPRESSOR OF PLASTID PROTEIN
350 IMPORT1 LOCUS1 (SP1) is a ubiquitin E3 ligase that is anchored to the chloroplast
351 outer envelope and induces proteasome-dependent degradation of some proteins of the
352 TOC (translocon on the outer chloroplast membrane) complex (Ling et al. 2012; Ling
353 and Jarvis 2015). To date, only two ubiquitin E3 ligases, PUB4 and SP1, were found to
354 be associated with the ubiquitination of chloroplasts. Eukaryotic genomes generally
355 encode large families of ubiquitin E3 ligases, and Arabidopsis can express more than
356 1,500 of these proteins based on genome-wide analysis (Vierstra 2012). Therefore,

another as yet unidentified ubiquitin E3 ligase might be involved in the induction of chlorophagy.

The regulation of chlorophagy during leaf senescence

Leaf senescence is a developmental process during which cytoplasmic components including chloroplasts undergo massive degradation and the released molecules are remobilized to newly developing organs. Photoinhibition may be enhanced during senescence, since photosynthetic activity decreases due to the degradation of photosynthetic proteins, and ROS accumulation is generally enhanced in senescing leaves (Juvany et al. 2013). Such enhanced ROS accumulation might activate chlorophagy during senescence.

However, entire chloroplasts were transported to the vacuole via chlorophagy at later stages of accelerated senescence in individual Arabidopsis leaves when covered with aluminum foil (Wada et al. 2009), which is an experimental condition widely used to analyze phenomena during leaf senescence. Under this condition, another type of chloroplast-targeted autophagy is preferentially activated, in which a portion of the chloroplast stroma is transported to the vacuole as a specific autophagic vesicle termed the Rubisco-containing body (RCB; Ishida et al. 2008; Izumi et al. 2015). Chloroplasts in covered senescing leaves are much smaller than those in young leaves; therefore, the active separation of stroma via RCBs are thought to result in chloroplast shrinkage, and these small chloroplasts are believed to become whole targets of autophagy (Izumi and Nakamura 2018).

In covered leaves that do not acquire light, photodamage does not occur; thus, the idea that senescence-induced chlorophagy and photodamage-induced chlorophagy are differentially regulated appears to be reasonable. In mammals, other forms of mitophagy distinct from the PINK1/Parkin-mediated type have been observed. In most mammals, red blood cells lack mitochondria due to the autophagic removal of mitochondria that accumulate the LC3-interacting protein, NIX (also known as BNIP3L), on the outer envelope (Schweers et al. 2007; Sandoval et al. 2008). This form of mitophagy is triggered by the upregulation of NIX expression during red blood cell differentiation. When $\Delta\Psi$ in mitochondria declines due to cell hypoxia, another LC3-interacting protein, FUN14 domain containing 1 (FUNDC1), accumulates on the outer envelope, thereby inducing mitophagy (Liu et al. 2012a). Hypoxia-induced dephosphorylation of FUNDC1 triggers this mitophagic process. Together, these findings suggest that in plants, chlorophagy might also be regulated by distinct mechanisms in different organ types, conditions or developmental stages.

Diverse pathways contribute to the degradation of intrachloroplastic components during leaf senescence without causing the digestion of entire chloroplasts via chlorophagy (Izumi and Nakamura 2018). In addition to the separation of stroma via the RCB pathway, chlorophylls are actively degraded through the autophagy-independent cascade via multi-step enzymatic reactions (Hortensteiner and Krautler 2011). Autophagy-independent routes that degrade stroma, thylakoid and envelope components during senescence include the formation of senescence-associated vacuoles, i.e., small vacuoles generated in the cytoplasm in senescing leaves (Martinez et al. 2008) and CHLOROPLAST VESICULATION-containing vesicles, a type of vesicle that

401 mobilizes a portion of the chloroplast into the vacuole (Wang and Blumwald 2014).
402 These active degradation processes of intrachloroplastic components might produce
403 almost empty chloroplasts that have lost photosynthetic activity. Therefore, it is also
404 conceivable that the same proteins that function during photodamage-induced
405 chlorophagy also recognize senescence-induced dysfunctional chloroplasts, however the
406 initial event that occurs during the induction of chlorophagy in both cases is distinct.

407 **Conclusions and Future Perspectives**

408 The discovery of photodamage-induced chlorophagy has prompted new questions,
409 including what types of chloroplast damage induce chlorophagy, how the damaged
410 chloroplasts are recognized and recruited to the core autophagy machinery and whether
411 photodamage-induced chlorophagy and senescence-induced chlorophagy share a
412 common regulatory mechanism (Fig. 2). Our summary of the process of photoinhibition
413 indicates that damage accumulates in PSII and PSI, which is manifested as ROS
414 accumulation and chloroplast envelope damage. Thus, investigating chlorophagic
415 activity in mutants of the respective systems that alleviate each type of damage may
416 help clarify the direct triggers of chlorophagy within photodamaged chloroplasts. Based
417 on studies of mitophagy in yeast and mammals, we postulate that unknown inducers and
418 autophagic receptors selectively recognize chloroplasts that exhibit specific types of
419 damage and recruit them as cargoes for chlorophagy (Fig. 2). Chloroplasts are
420 approximately 5–7 μm in diameter, which is much larger than mitochondria and typical
421 autophagosomes, which are only approximately 1 μm in diameter (Yoshimoto et al.
422 2004; Thompson et al. 2005). How chloroplasts are incorporated into the vacuole, i.e.,

via macroautophagy, microautophagy or other pathways, is another fascinating issue to uncover (Fig. 2).

Elucidation of the chlorophagy induction mechanism is still in the initial stages. To improve our understanding of this mechanism, additional studies should investigate chloroplast function and compare organelle-selective autophagy among different eukaryotes.

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Conflicts of Interest

The authors declare no conflicts of interest.

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635
636 **Legends to Figures**

637 **Figure 1.** Images and schematic representation of chlorophagy induced by strong
638 visible light in Arabidopsis. (A) Confocal images of leaf mesophyll cells expressing
639 stroma-targeted GFP under the control of the *35S* promoter. The second rosette leaves of
640 non-treated control plants or plants at 2 d after exposure to 2 h of high visible light (HL;
641 2,000 μmol m^{-2} s^{-1}) at 10°C were observed. Arrowheads indicate chloroplasts lacking
642 stroma-localized GFP. Chlorophyll autofluorescence appears magenta, and GFP signals
643 appear green. In the merged images, overlapping regions of chlorophyll and GFP appear
644 white. Differential interference contrast (DIC) images are also shown. Scale bars = 10
645 μm. (B) Confocal images of leaf mesophyll cells expressing tonoplast-targeted GFP-
646 delta tonoplast intrinsic protein (δTIP) under the control of the *35S* promoter and
647 stroma-targeted Rubisco small subunit (RBCS)-RFP under the control of the *RBCS*
648 promoter. The second rosette leaves of non-treated control plants or plants 1 d after
649 exposure to 2 h HL at 10°C were observed. Arrowheads indicate chloroplasts in the
650 vacuolar lumen. Chlorophyll autofluorescence appears magenta. In the merged images,
651 GFP and RFP signals appear green. DIC images are also shown. Scale bars = 10 μm.
652 (C) TEM images of leaf mesophyll cells from wild-type plants. The second rosette
653 leaves of non-treated control plants or plants 1 d after exposure to 2 h HL at 10°C were

fixed and observed. Images in the right panels are enlargements of the boxed regions in the left. Scale bars = 5 μm. Arrowheads indicate vacuolar chloroplasts resulting from chlorophagy. (D) Schematic model of photodamage-induced chlorophagy. In this model, photodamaged chloroplasts are transported into the vacuolar lumen for degradation via autophagic membrane-associated sequestration.

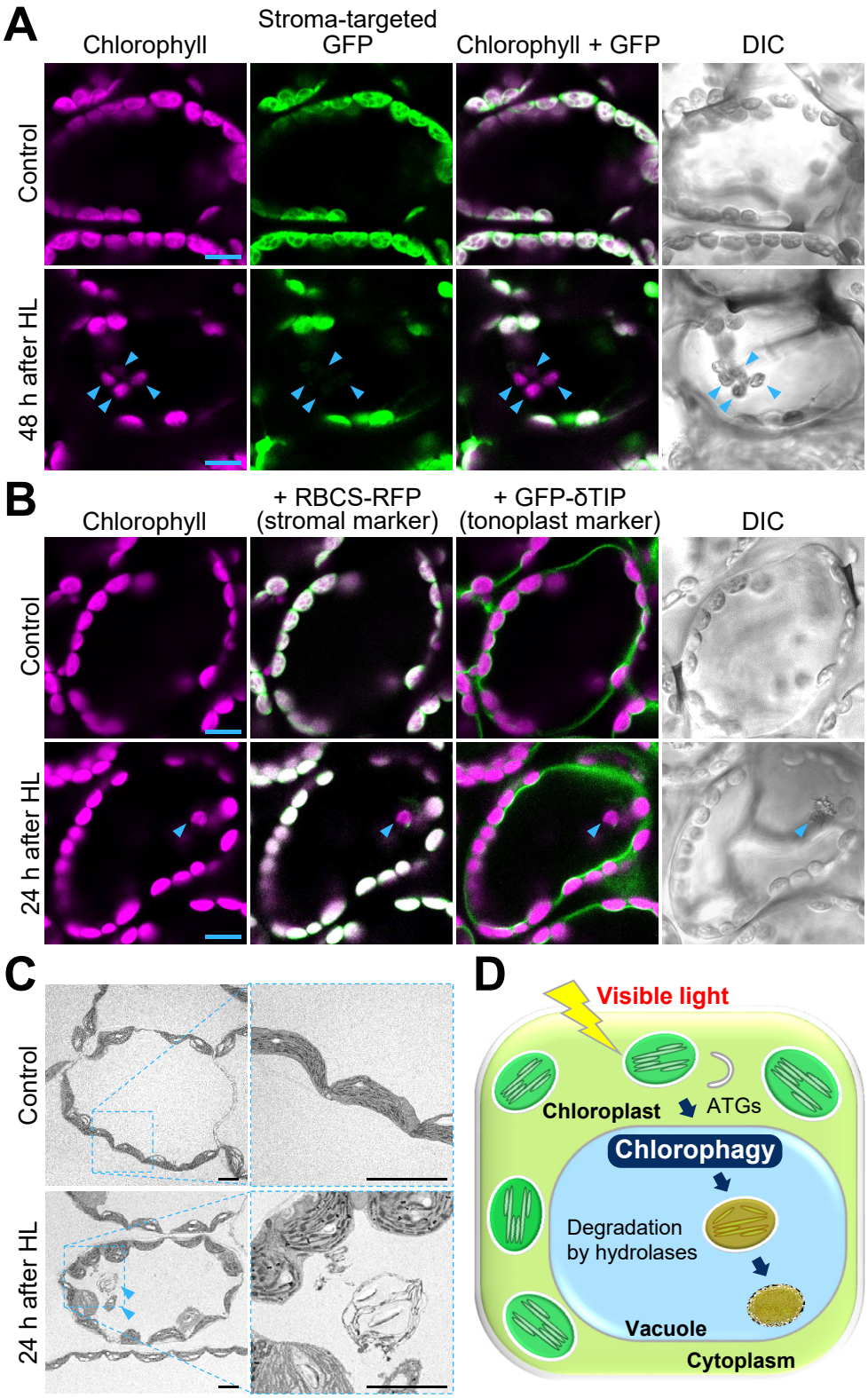
Figure 2. Possible mechanism for the regulation of chlorophagy: lessons from mitophagy regulatory mechanisms in mammals.

(A) Possible events leading to photodamage-induced chlorophagy. Plant chloroplasts can accumulate several types of damage during photoinhibition, including PSII and PSI damage, ROS accumulation and envelope damage. Specific types of damage within the chloroplast might act as a direct trigger of chlorophagy. Based on our understanding of mitophagy in mammals (shown in B), unknown proteins that interact with targeted chloroplasts might act as inducers or autophagic receptors for chlorophagy. Outer envelope-associated proteins or ubiquitins might be involved in this induction process. How chloroplasts are incorporated into the vacuole remains unknown.

(B) Schematic models of the events leading to three types of selective mitophagy mechanisms in mammalian cells. (a) PINK1/Parkin-mediated mitophagy is initiated upon the accumulation of PINK1 on the outer membranes of depolarized mitochondria. PINK1 then phosphorylates ubiquitin to activate the E3 ligase, Parkin, thereby leading to the accumulation of ubiquitin chains on the outer envelope. Several types of autophagic receptors that bind to LC3 (a mammalian homolog of ATG8), including NDP52, optineurin and p62, interact with ubiquitinated mitochondrial proteins and

autophagosome-anchored LC3, which induces the sequestering of depolarized mitochondria by the autophagosome. (b) NIX acts as a mitophagy receptor that directly binds to LC3 on the outer envelope to induce mitophagy during red blood cell differentiation. This phenomenon is triggered by the upregulation of NIX expression. (c) Dephosphorylation of FUNDC1 on the mitochondrial outer envelope in response to hypoxia allows the protein to directly interact with LC3, thereby inducing mitophagy.

Fig. 1



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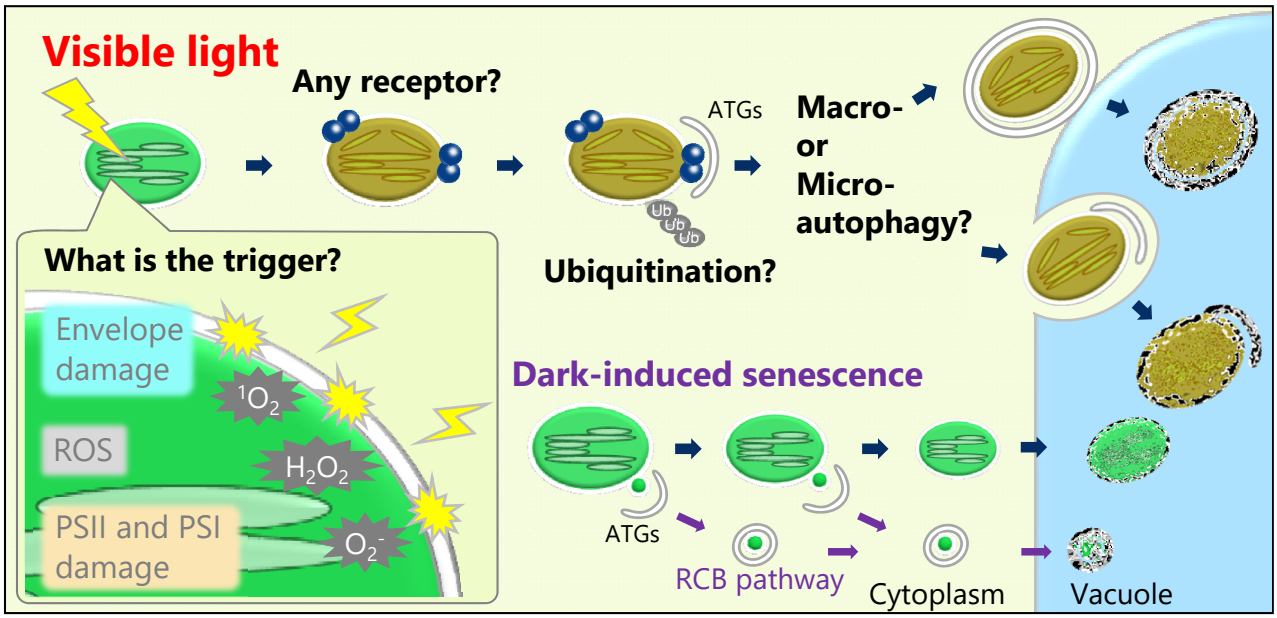
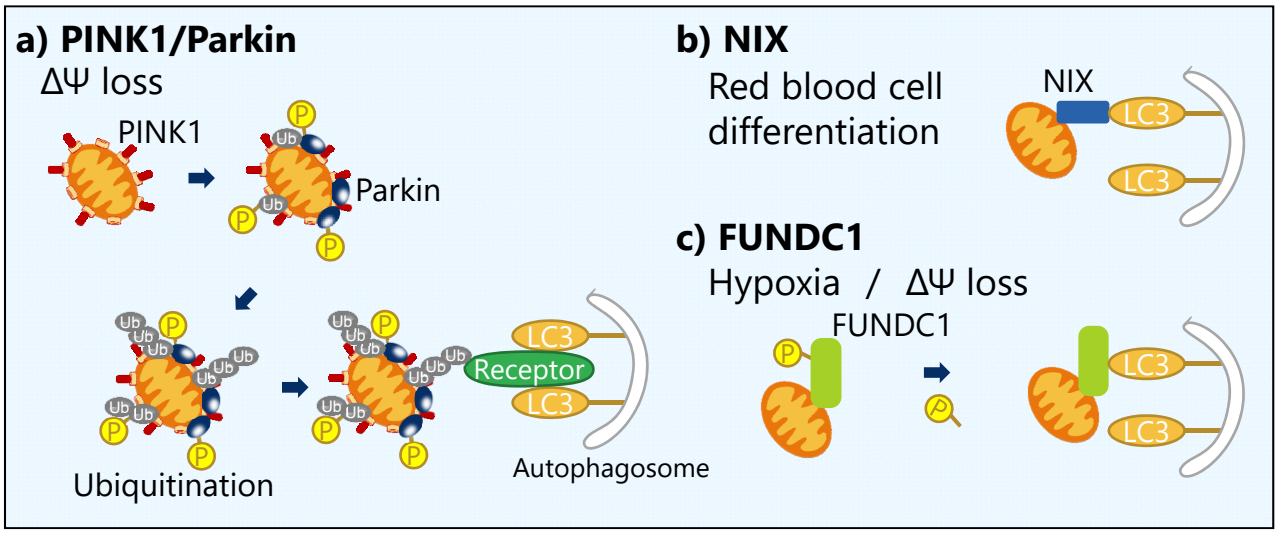
(A) Confocal images of leaf mesophyll cells expressing stroma-targeted GFP under the control of the *35S* promoter. The second rosette leaves of non-treated control plants or plants at 2 d after exposure to 2 h of high visible light (HL; 2,000 μmol m^{-2} s^{-1}) at 10℃ were observed. Arrowheads indicate chloroplasts lacking stroma-localized GFP. Chlorophyll autofluorescence appears magenta, and GFP signals appear green. In the merged images, overlapping regions of chlorophyll and GFP appear white. Differential interference contrast (DIC) images are also shown. Scale bars = 10 μm. (B) Confocal images of leaf mesophyll cells expressing tonoplast-targeted GFP-delta tonoplast intrinsic protein (δTIP) under the control of the *35S* promoter and stroma-targeted Rubisco small subunit (RBCS)-RFP under the control of the RBCS promoter. The second rosette leaves of non-treated control plants or plants 1 d after exposure to 2 h HL at 10℃ were observed. Arrowheads indicate chloroplasts in the vacuolar lumen. Chlorophyll autofluorescence appears magenta. In the merged images, GFP and RFP signals appear green. DIC images are also shown. Scale bars = 10 μm. (C) TEM images of leaf mesophyll cells from wild-type plants. The second rosette leaves of non-treated control plants or plants 1 d after exposure to 2 h HL at 10℃ were fixed and observed. Images in the right panels are enlargements of the boxed regions in the left. Scale bars = 5 μm. Arrowheads indicate vacuolar chloroplasts resulting from chlorophagy. (D) Schematic model of photodamage-induced chlorophagy. In this model, photodamaged chloroplasts are transported into the vacuolar lumen for degradation via autophagic membrane-associated sequestration.

Fig. 2

A. Chlorophagy in plant cells



B. Mitophagy in mammalian cells



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