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Review

Roles of autophagy in chloroplast recycling ☆

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ABSTRACT

Chloroplasts are the primary energy suppliers for plants, and much of the total leaf nitrogen is distributed to these organelles. During growth and reproduction, chloroplasts in turn represent a major source of nitrogen to be recovered from senescing leaves and used in newly-forming and storage organs. Chloroplast proteins also can be an alternative substrate for respiration under suboptimal conditions. Autophagy is a process of bulk degradation and nutrient sequestration that is conserved in all eukaryotes. Autophagy can selectively target chloroplasts as whole organelles and or as Rubisco-containing bodies that are enclosed by the envelope and specifically contain the stromal portion of the chloroplast. Although information is still limited, recent work indicates that chloroplast recycling via autophagy plays important roles not only in developmental processes but also in organelle quality control and adaptation to changing environments. This article is part of a Special Issue entitled: Dynamic and ultrastructure of bioenergetic membranes and their components.

1. Introduction

Chloroplasts are the characteristic organelles of plants and photoautotrophs. In addition to performing photosynthesis, chloroplasts are central to plant metabolism. For example, assimilation of nutrients and biosynthesis of various metabolites such as amino acids, fatty acids, pigments and hormones, occur in chloroplasts. During the vegetative growth stage, the majority of plant nitrogen and other nutrients are distributed to leaves [1,2]. Further, approximately 80% of the total leaf nitrogen is found within chloroplasts, mainly as photosynthetic proteins, in C_3 plants [3]. Around 70% of chloroplast nitrogen is present in the stroma and the remaining portion is in the thylakoid membrane. The chloroplast carbon-fixing enzyme Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) is exceptionally abundant, accounting for 10 to 30% of the leaf nitrogen [4,5]. In addition, around 7% of leaf nitrogen is found in the light-harvesting chlorophyll a/b protein of photosystem II (LHCII) in the thylakoid membrane [5].

Because plants are sessile, efficient use and recycling of assimilated nutrients are particularly important for their survival and fitness under ever-changing environments. Senescence represents the final developmental stage and a form of programmed cell death in each organ of plants. Leaf senescence can be viewed as primarily a process through which cellular macromolecules are actively degraded and their components are remobilized. During leaf senescence, Rubisco and other chloroplast proteins are gradually degraded as a major source of nitrogen for recycling, and this degradation correlates with a decline in photosynthetic activity [6–8]. Additionally, these proteins can be degraded under starvation conditions caused by darkness, with their carbon skeletons serving as substrates for respiration [6,9]. As the levels of the inner components decline, chloroplasts gradually shrink and transform into gerontoplasts, in which thylakoid membranes are disintegrated and plastoglobules accumulate, and the cellular population of chloroplasts concomitantly declines.

The vacuole in leaf mesophyll cells occupies as much as 80% to over 90% of the total cell volume and is rich in a wide range of lytic hydrolases [10]. In fact, most or all of the proteolytic activity against Rubisco has been found in the vacuolar fraction [11], and several vacuolar cysteine proteases are induced in a senescence-associated manner [12]. Studies in the early 1980s led to the proposal that sequential degradation of chloroplasts within the vacuole serves as the major pathway for chloroplast protein degradation in senescing leaves [11,13]. Under electron microscopy, chloroplasts appear to be either within the vacuole or within invaginations of the tonoplast in mesophyll protoplasts isolated from wheat (*Triticum aestivum*) leaves undergoing darkness-induced senescence [13]. Based on those findings, the authors proposed that chloroplasts were taken up in the vacuole by 'a phagocytic-type mechanism'. The process of delivery of cytoplasmic components such as proteins and organelles to the vacuole for degradation is now widely recognized as autophagy.

The decline in Rubisco protein levels is much faster than that of chloroplast population size during senescence [8,14,15]. Similarly, the decreases in major chloroplast proteins do not proceed in parallel; Rubisco decreases faster than does LHCII, for example [16,17]. These two proteins

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are synthesized mainly during leaf expansion and turn over less after that [18,19]. These results indicate that alternative pathways or mechanisms other than autophagy of entire chloroplasts must function in Rubisco degradation. Accordingly, much effort in the study of Rubisco degradation has been focused on chloroplast proteases rather than autophagy [20–23]. It is now evident that a number of proteases inside chloroplasts play crucial and diverse roles in chloroplast development and maintenance. Recent genome-wide studies have revealed the existence of proteases of prokaryotic origin in chloroplasts. Of these, ATP-dependent proteases such as Clp, FtsH, and Lon are considered to be the major enzymes involved in the gradual degradation of proteins into oligopeptides and amino acids [24]. Some of these proteases could play important roles in the senescence-associated bulk degradation of chloroplast proteins, but whether this is the case remains controversial [25,26].

2. Autophagy

2.1. Lessons from yeast

Autophagy is an evolutionarily conserved system for bulk degradation of intracellular components in eukaryotes. The mechanism of autophagy has been particularly well studied in yeast species, in which cytosol, sometimes including entire organelles, is engulfed in membrane-bound vesicles [27]. These vesicles are delivered to lytic compartments, namely vacuoles or lysosomes, where the vesicles and their contents are degraded by a variety of resident hydrolases. Two morphologically distinct forms of autophagy, called microautophagy and macroautophagy, have been observed in wide range of organisms including higher plants [28]. During microautophagy, cytoplasmic components are directly engulfed by an invaginated vacuolar membrane. In macroautophagy, which is the major pathway and hereafter is referred to simply as autophagy, the cytosol is sequestered into a double-membraned vesicle called an autophagosome. The outer membrane of the autophagosome then fuses to the vacuolar membrane, thereby delivering the inner membrane-delimited structure, the autophagic body, into the vacuolar lumen.

Autophagy can be selective or non-selective in terms of substrate targeting [29]. During starvation-induced autophagy, cytoplasmic components are generally considered to be non-selectively engulfed by autophagosomes, although some cytoplasmic proteins are known to be preferentially targeted [30,31]. By contrast, peroxisomes and mitochondria are selectively targeted for autophagy during pexophagy and mitophagy, respectively. In addition, budding yeast (*Saccharomyces cerevisiae*) exhibits a type of autophagy, referred to as the Cvt (cytoplasm-to-vacuole targeting) pathway, that mediates the biosynthetic transport of abundant vacuolar proteins such as aminopeptidase I (Ape I). Genetic studies in yeast have identified more than 30 *AuTophaGy-related genes* (*ATGs*) that participate in autophagic processes. Among these *ATGs*, 15 (*ATG1-10*, *12-14*, *16*, and *18*) are commonly required for all of the above-described autophagic pathways and are referred to as 'core' *ATGs* [27,29]. The core *ATG* products together with vacuolar protein sorting 34 (Vps34) and Vps15 constitute the fundamental 'core machinery' responsible for the biogenesis of autophagy-related membranes. The core *ATG* products can functionally be classified into subgroups: the Atg1 kinase complex (Atg1, Atg13), the autophagy-specific phosphatidylinositol 3-kinase (PI3K) complex (Atg6, Atg14), the Atg9 complex (Atg2, Atg9, Atg18), and two ubiquitin (Ub)-like conjugation systems, the Atg12 conjugation system (Atg5, Atg7, Atg10, Atg12, Atg16) and the Atg8 lipidation system (Atg3, Atg4, Atg7, Atg8). The detailed functions of the core *ATG* products have been summarized well in recent reviews [27,32,33]. Most core *ATG* products are also essential to microautophagy [34–36].

In addition to these core components, other Atg proteins are specifically required for different subtypes of autophagy. In the Cvt pathway, precursor Ape1 is packed into a Cvt complex along with the cargo receptor Atg19, and the adapter protein Atg11 mediates the delivery of the Cvt complex to the pre-autophagosomal structure (PAS), where the Cvt vesicle is formed [29,37,38]. Atg11 is also important for pexophagy and mitophagy [37,39]. During starvation-induced autophagy, Atg17, Atg29, and Atg31 are essential as regulators to form the Atg1 kinase complex, which is required for the induction of autophagosome formation. Here, the target of rapamycin (TOR) kinase is known to be an upstream negative regulator of the Atg 1 complex [40,41]. Under nutrient-rich conditions, the TOR complex 1 (TORC1) hyperphosphorylates Atg13, promoting the dissociation of the Atg1 kinase complex. Under starvation conditions, TORC1 is inactivated, Atg13 is rapidly dephosphorylated, the Atg1 complex is formed, and autophagy is induced.

2.2. Autophagy in plants

Plant autophagy has long been studied by morphological observation using microscopy [33]. More recently, initial genome-wide studies opened the door to molecular analysis of plant autophagy by identifying a number of genes homologous to yeast *ATGs* and some of their knockout mutants in Arabidopsis (*Arabidopsis thaliana*) [42,43]. Although Arabidopsis does not have homologs of the subtype-specific *ATGs* [44], the Arabidopsis genome contains homologs of almost all core *ATGs* identified in yeast [33,45]. The only exception is ATG14, which is also missing in rice (*Ozyza sativa*) and *Chlamydomonas* (*Chlamydomonas reinhardtii*) [45]. Several Arabidopsis *ATG* homologs are present in multiple copies, and some of those are functionally redundant [46,47]. The roles of Arabidopsis *ATGs* have been studied using T-DNA insertional knock-out mutants and RNA interference knockdown mutants. In addition, a live-cell system for monitoring autophagy in plants was established using a green fluorescent protein (GFP)-ATG8 fusion protein as a marker for autophagosomes [46,48,49]. These molecular approaches have confirmed that the core machinery for autophagy functions in plants as it does in yeast and also revealed the importance of plant autophagy in responses to nutrient starvation, abiotic stresses, and pathogen infection [42,43,46–48,50–56]. It has also been shown that TOR kinase serves as a negative regulator in Arabidopsis as in yeast [57].

The response to nutrient starvation was an initial focus of autophagy studies in plants, as it represents a central function of autophagy in yeast. Autophagy is induced when cultured plant cells are deprived of exogenously supplied sucrose [58–60]. Under those conditions, core *ATGs* related to Ub-like conjugation systems are transiently up-regulated [61]. The role of autophagy in nutrient recycling has also been studied using autophagy-deficient (*atg*) mutant plants. In principle, the Arabidopsis *atg* mutants can complete their life cycles [42,43]. However, *atg* mutants cannot survive for long periods under nitrogen- and/or carbon-starvation conditions. They also show accelerated leaf senescence and cell death, concomitant with reductions in chlorophyll and photosynthetic proteins, even under favorable nutrient and growth conditions. As a result of such premature senescence, seed production appears to decrease in *atg* mutants. Based on the phenotypes of the *atg* mutants, it was concluded at the time that although autophagy is important in nutrient recycling during starvation and senescence in plants, chloroplasts, despite being the most abundant degradation substrates in leaves, were not main targets for autophagy [28,62].

3. Piecemeal degradation of chloroplasts via Rubisco-containing bodies and senescence associated vacuoles

The specific release of Rubisco from chloroplasts and its subsequent degradation in other compartments has been proposed as an explanation for the earlier decline in Rubisco compared to the chloroplast population [8]. In the case of Chlamydomonas, some Rubisco is localized to the vacuole and a protrusion of the outer membrane of the envelope enclosing the stroma is sometimes observed, possibly accounting for exclusion of Rubisco from chloroplasts [63]. Senescing chloroplasts accumulate plastoglobules, which are thylakoid-associated monolayer

particles containing lipids and proteins [64]. Plastoglobules can protrude through the chloroplast envelope and emerge into the cytoplasm in senescing soybean (*Glycine max*) leaves [65]. Those reports support the existence of pathways that transport chloroplast components to the cytoplasm or to the vacuole for degradation before the destruction of entire chloroplasts.

As previously summarized [66], it is currently clear that at least two distinct transport pathways are responsible for the extra-chloroplastic degradation of stromal proteins: *ATG*-dependent autophagy via Rubisco-containing bodies (RCBs) for degradation in the central vacuole and an *ATG*-independent alternative pathway involving senescence-associated vacuoles (SAVs) (Fig. 1).

3.1. Rubisco-containing bodies

RCBs were originally identified in naturally senescing leaves of wheat (*T. aestivum*) [17]. Detailed immunoelectron-microscopic observations revealed that Rubisco is sometimes localized in small spherical bodies (RCBs) that are located mostly in the cytoplasm and occasionally in the vacuole. RCBs contain another stromal protein, Gln synthetase, and have an electron-staining density similar to that of chloroplast stroma. RCBs do not contain thylakoid structures or the major membrane proteins such as LHCII, the α and β-subunits of coupling factor 1 of ATPase, or cytochrome *f*. The double membranes of RCBs seem to derive from the chloroplast envelope, and RCBs are further surrounded by other membrane structures such as isolation membranes (phagophores) in the cytoplasm. RCBs are frequently found in the early phase of leaf senescence when the amount of Rubisco starts to decrease but chlorophyll still remains constant.

Piecemeal degradation of chloroplasts via RCBs seems to be common among plants, and it is important for protein recycling in both development and abiotic stress responses. RCBs are also observed in young leaves of tobacco (*Nicotiana tabacum*), where they have been referred to as Rubisco-vesicular bodies (RVBs) [67], and in leaves of rice (*Oryza sativa*) under salt stress conditions [68]. The RCBs found in rice leaves under salt stress have two distinct structural features compared to previously described RCBs. The rice RCBs have inner membrane structures, the formation of which could be related to the vesicles derived from invagination of the chloroplast inner envelope. In addition, rice RCBs sometimes contain the crystalline inclusions that are formed in chloroplasts under osmotic stress and disappear during recovery. It is possible that RCBs are responsible for the degradation of these crystalline inclusions. Potential processes involved in RCB formation during salt stress have been illustrated in detail [68].

The relationship between RCBs and autophagy has been revealed with the aid of reverse genetics and live-cell imaging techniques in Arabidopsis. RCBs can be visualized using stroma-targeted GFP (or RFP) or a small subunit of Rubisco (RBCS)-GFP (or RFP) fusion that interacts with endogenous large subunit of Rubisco (RbcL) and RBCS molecules to form Rubisco-GFP (or RFP) in the plant [69]. When

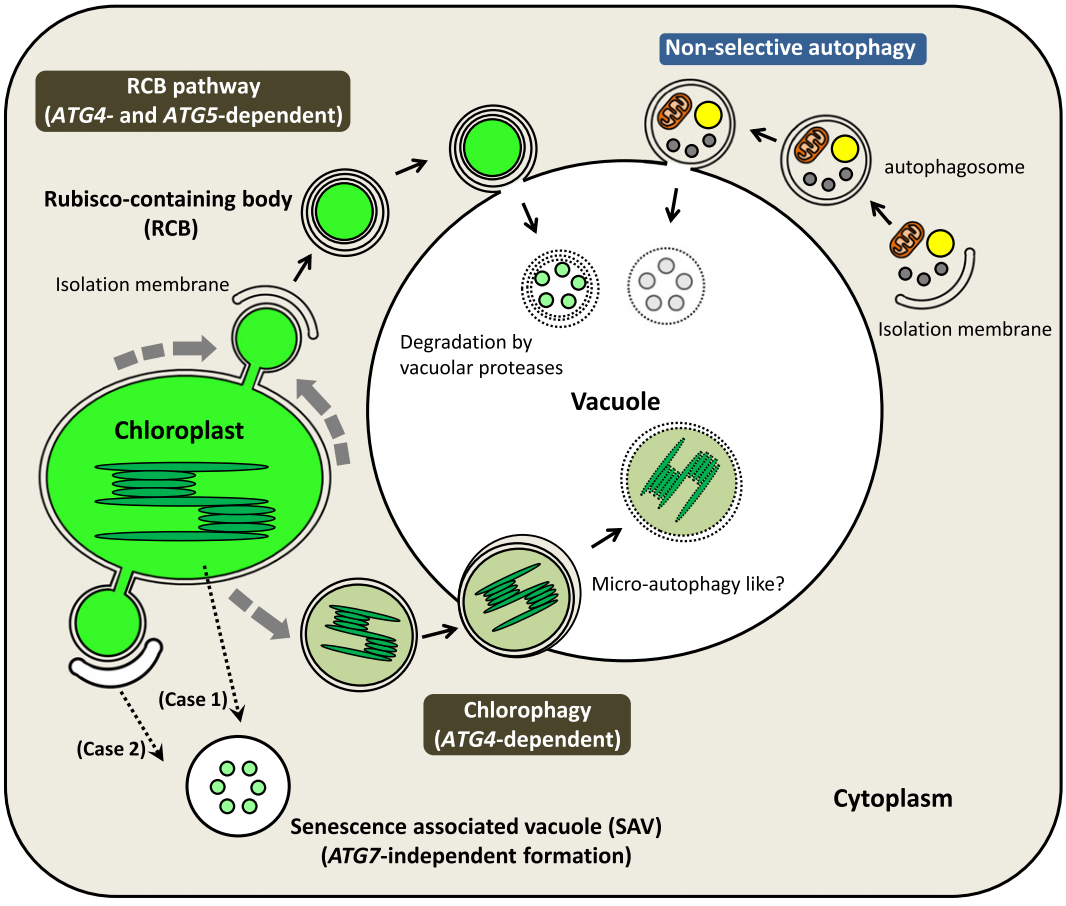

Fig. 1. Pathways for vacuolar degradation of chloroplasts and their proteins. In addition to non-selective autophagy of cytoplasmic components and organelles, chloroplasts can be selectively degraded via a number of different pathways. In the RCB pathway, a chloroplast protrusion may be sequestered by an isolation membrane. The resultant Rubisco-containing body (RCB), an autophagosome specifically containing stromal proteins enclosed by the chloroplast envelope, is transported into the central vacuole by *ATG4*- and *ATG5*-dependent autophagy. The RCB is then degraded by vacuolar proteases. The remaining chloroplast, shrunken by the production of RCBs, is then transported into the central vacuole via an *ATG4*-dependent, possibly microautophagy-like, process (chlorophagy). Alternatively, stromal proteins may be transported to a senescence-associated vacuole (SAV) by an as yet unknown direct mechanism (Case 1) or by sequestration of a part of the chloroplast or stromule (Case 2). The formation of SAVs is *ATG7*-independent.

Arabidopsis leaves expressing those fluorescent markers are incubated with concanamycin A to suppress vacuolar lytic activity, spherical bodies exhibiting GFP or RFP fluorescence without chlorophyll fluorescence, namely RCBs, are observed in the vacuolar lumen [69]. In addition, RCB accumulation is disrupted in the autophagy-defective mutants, *atg5* and *atg4a4b* [69,70]. In wild-type cells, stroma-targeted RFP and the GFP-ATG8a fusion, a marker for the autophagosome and autophagic bodies, are observed together in autophagic bodies in the vacuole [69,71]. These results support the conclusion that RCBs are a type of autophagic body specifically containing Rubisco and possibly other stroma-localized proteins (Fig. 1; [69]).

3.2. Possible mechanisms for RCB formation

How RCBs form has yet not been elucidated. Whole organelles such as mitochondria and peroxisomes can be entirely engulfed by the vacuole during microautophagy or entirely sequestered into autophagosomes which then fuse with the vacuole. By contrast, the endoplasmic reticulum (ER) is subject to the piecemeal type of organelle autophagy. The ER is dynamic and comprises a large three-dimensional network of continuous tubules and sheets bound by a single membrane. The ER is composed of rough ER (rER), smooth ER (sER) and the nuclear envelope [72]. Rough ER containing the DsRed marker fused to the HDEL ER retention signal is partially engulfed by autophagosomes under starvation conditions in yeast [73]. Autophagosomes cannot engulf the ER in the presence of Latrunculin A, which disrupts actin structures and blocks the dynamics of the ER network. It has been suggested that the dynamics of the ER network lead to transient formation of ER fragments in the cytoplasm and that these fragments can then be engulfed by autophagosomes [73]. Recently, this partial engulfment of the rough ER has also been demonstrated to occur in Arabidopsis during ER stress [74].

The nuclear envelope including a portion of the nucleus is engulfed by the vacuole in yeast, a phenomenon that was originally referred to as 'piecemeal microautophagy' of the nucleus (PMN) [75]. In yeast, Velcro-like patches forming nucleus-vacuole junctions are generated through specific interactions between Vac8p on the vacuole membrane and Nvj1p in the nuclear envelope [76]. PMN at nucleus-vacuole junctions results in the pinching-off and release into the vacuole of nonessential portions of the nucleus [75]. PMN occurs in rapidly dividing cells but is induced to higher levels by carbon and nitrogen starvation. In response to nutrient depletion, Nvj1p increasingly binds and sequesters two proteins with roles in lipid metabolism, Osh1p and Tsc13p [77]. In addition to these specific components, PMN requires the set of core Atgs [35].

Stroma-filled tubules (stromules) may have a functional role in piecemeal autophagy of chloroplasts. Stromules are thin extensions of the stroma surrounded by a double envelope membrane and emanate from plastid bodies. They are highly dynamic, branching and elongating across the plant cell [78], and occasionally fragmenting and releasing small vesicles [79]. The autophagosome marker GFP-ATG8 is sometimes observed on the chloroplast surface [47] and in a chloroplast protrusion [69,80] which might be an incipient stromule. Stromules are abundant on chlorophyll-free plastids such as those in roots, petals, and suspension-cultured cells and are rarely seen on mesophyll chloroplasts [81,82]. However, stromules can be frequently found in mesophyll chloroplasts in *atg5* mutants after dark-induced starvation [69] or natural senescence (Fig. 2). It is possible that chloroplast protrusions and stromules cannot be sequestered by isolation membranes in *atg5* cells, consequently leading to an increase in stromule length and frequency.

Stromules closely associate with the ER, the nucleus, and mitochondria [83,84]. A recent live-cell imaging study using fluorescent proteins clearly showed coincidental behavior of stromules and the ER [84]. These results suggest that either the neighboring ER tubules shape stromules directly or the behavior of both ER and stromules is simultaneously dictated by a shared cytoskeleton-based mechanism. As the ER harbors the largest reservoir of cellular membranes, it is highly likely that it is recycled by autophagy during senescence as during starvation conditions and under ER stress [46,74]. The presence of complex stromules in autophagy-deficient cells may reflect the accumulation of surplus membranes of both chloroplasts and the ER and their close interactions.

3.3. Senescence-associated vacuoles

A novel type of small lytic vacuole referred to as the senescence-associated vacuole (SAV) has been identified in the cytoplasm of

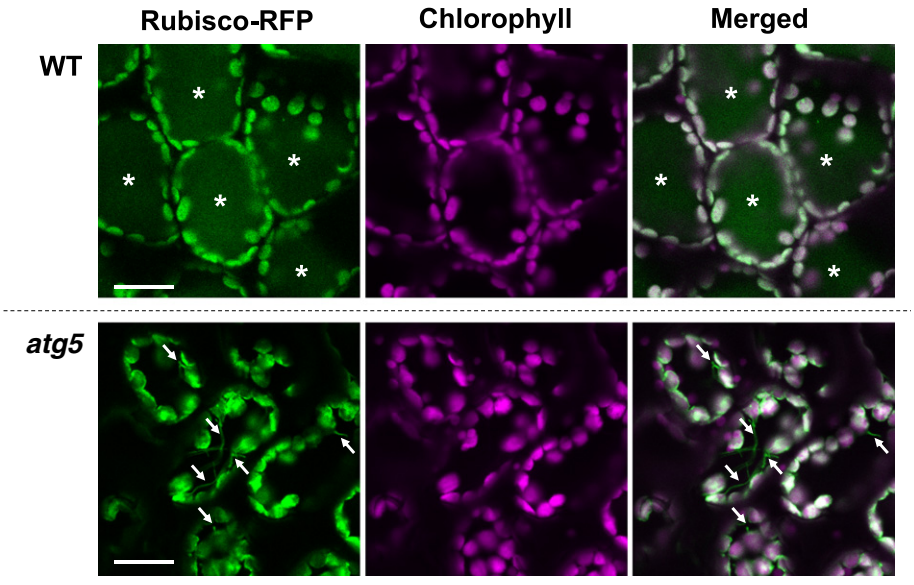

Fig. 2. Visualization of Rubisco-RFP fluorescence in living mesophyll cells of naturally-senescent wild-type (WT) and *atg5* Arabidopsis. Plants were grown for 50 days in soil culture under long day conditions. A senescent rosette leaf excised from the plant was observed immediately by laser scanning confocal microscopy. RFP fluorescence appears pseudo-colored green and chlorophyll autofluorescence is magenta. In merged images, overlapping signals appear white. In addition to chloroplasts, the RFP fluorescence is observed in the vacuolar lumen (asterisks) in wild-type, but not in the *atg5* mutant. Instead, many stromules and chloroplast protrusions (arrows) are observed in the *atg5* mutant. Bars = 25 μm.

senescent leaves of soybean (*Glycine max*), Arabidopsis, and tobacco as an alternative extra-chloroplastic pathway for chloroplast protein degradation [85,86]. SAVs are found only in senescing photosynthetic tissues and contain a senescence-specific cysteine-protease, SAG12 (senescence-associated gene 12) [85]. Similar to RCBs, SAVs contain stromal proteins such as Rubisco and Gln synthetase, and stromal-targeted GFP, but do not contain thylakoid proteins such as LHCII. SAVs also contain chlorophyll *a*, whereas RCBs do not. To date, there is no evidence that SAVs use any autophagic machinery. Importantly, it was stated that SAVs still formed in the autophagy-defective *atg7* mutant, although the data were not shown in the original article [85]. This indicates that the contents of RCBs and SAVs are similar, but that the mechanisms of their formation are clearly distinct. A recent study showed that SAV accumulation is concomitant with induction of autophagy in the Arabidopsis *des1* mutant, which lacks L-Cys desulfhydrase 1 for the degradation of cysteine [87]. Further studies are required to clarify the relationship between *ATG*-dependent autophagy and SAVs in detail.

The morphology of SAVs is markedly different from that of RCBs (compare Fig. 3 in ref. [85] with Fig. 5 in ref. [17]). As described above, RCBs are surrounded by a double membrane and their interior has similar electron density to the stroma of chloroplasts. By contrast, SAVs have a single membrane. The electron density of the inner space of SAVs is similar to that of the central vacuole and is much lower than that of the chloroplast stroma. In addition, SAVs often contain dense aggregates in their lumen that may consist of partially degraded cellular materials.

How stromal proteins are targeted to SAVs is currently uncertain. As presented in a previous review [88], stromal proteins might cross the chloroplast envelope and then be directly transferred to SAVs through an as yet unknown mechanism (Case 1 in Fig. 1). Alternatively, it is possible that, as in the piecemeal microautophagy of the nucleus in yeast, the SAV itself sequesters a part of the chloroplast or stromule, forming a RCB-like particle (Case 2 in Fig. 1). SAVs are more acidic than the central vacuole and contain very strong proteolytic activities. Thus, even in the latter case (Case 2), sequestered particles of chloroplasts would be rapidly degraded inside SAVs, similar to the case of RCBs in the central vacuole, which can be visualized only in the presence of concanamycin A.

4. Chlorophagy — autophagy of whole chloroplasts

In the late stage of senescence, the number of chloroplasts decreases. Electron-microscopic studies have suggested the existence of whole-chloroplast autophagy, termed chlorophagy, in dark-induced senescing leaves [13,89]. In Arabidopsis, when a leaf of a plant is individually darkened, senescence is rapidly induced and both the number and the size of chloroplasts significantly decrease within few days [90,91]. In individually-darkened leaves (IDLs) of the *atg4s* mutant, visible senescence (i.e. chlorosis) is promoted as in wild-type, but the decrease in chloroplast number and, in part, the decrease in chloroplast size are impaired [70]. In addition to RCBs, small chloroplasts retaining chlorophyll fluorescence are observed within the vacuole after 3 days of the IDL treatment in wild type but not in the *atg4* mutant. As RCB formation consumes a portion of both the stroma and the chloroplast envelope, it is likely responsible for the reduction in chloroplast size. The resulting shrunken chloroplasts, possibly like gerontoplasts, are transported into the vacuole by autophagy (Fig. 1; [70]).

Mutations impairing chloroplast functions can cause the degradation of whole chloroplasts, possibly by chlorophagy, in a senescence- and starvation-independent manner. Partially-degraded chloroplasts are observed in the vacuole in cotyledon cells of the Arabidopsis *ppi40* (*plastid protein import Tic40*) mutant, which lacks a homologue of the 40 kDa protein of the pea translocon at the inner envelope membrane of chloroplasts (Tic complex) [92]. As vacuolar transfer of chloroplasts is observed under non-starved conditions, the authors proposed that plants can remove abnormal plastids by autophagy under nutrient-sufficient conditions for quality control of organelles. The Arabidopsis *mex1* (*maltose excess 1*) mutant, which lacks the maltose transporter in the chloroplast envelope, accumulates high levels of maltose and starch in chloroplasts and shows reduced numbers of chloroplasts and a chlorotic phenotype at the non-senescing stage [93,94]. Chloroplast components such as thylakoid membranes, starch granules, and plastoglobules are observed within the vacuole of the *mex1* mutant [94]. These findings led the authors to hypothesize that the accumulation of maltose in the *mex1* mutant causes chloroplast dysfunction, which may be signaled via a form of retrograde signaling and trigger chloroplast degradation. Whether vacuolar degradation of impaired chloroplasts is *ATG* dependent has not been elucidated yet.

5. Selectivity of chloroplast-targeted autophagy

Recent studies have shown that there are several types of selective autophagy in plants [32,33,95]. The first reported case was that of protein aggregates of overexpressed cytochrome b5-RFP fusion, which are degraded by autophagy preferentially over other marker proteins of the ER, mitochondria, and chloroplasts in tobacco suspension-cultured BY-2 cells [96]. Although the molecular mechanisms underpinning the process are currently unknown, there is evidence that chloroplast-targeted autophagy has the potential to be selective.

5.1. RCB versus non-selective autophagy

RCBs are defined as specific autophagic bodies that contain the stromal portion of chloroplasts but do not target thylakoid membranes and their constituents such as chlorophylls and membrane-integrated proteins. Stromules do not contain plastid nucleoids or ribosomes [97], suggesting that RCBs also have no such structures. The mechanism of selectivity of RCBs for stromal proteins is unclear. The ratio of Rubisco to plastid stromal-targeted GFP in RCBs seems to be indistinguishable from that in the stroma itself (see Fig. 3 in ref. [69]).

The induction of the RCB pathway is controlled differently than that of non-selective (non-RCB-type) autophagy (Fig. 1; [71]). Non-selective autophagy is up-regulated under both nitrogen- and carbon-limited conditions in plants. However, RCB autophagy is specifically linked to leaf carbon status rather than nitrogen status in Arabidopsis [71]. In excised leaves, RCB production is strongly suppressed by metabolic sugars, either externally supplied or internally produced via photosynthesis in the light, but it is not suppressed by externally-supplied inorganic nitrogen. Unlike RCBs, the production of non-RCB-type autophagic bodies is not suppressed in the light but is suppressed by exogenous inorganic nitrogen [71]. In plants, when the inorganic nitrogen supply is cut off, leaf carbohydrates are accumulated and RCB production is suppressed [71].

5.2. RCB versus whole organelle autophagy

It is particularly interesting that plants use two distinct autophagy pathways for chloroplasts, the RCB pathway and chlorophagy (Fig. 1). This combination of pathways may be important to facilitate complete degradation of large organelles. Autophagic bodies in plant cells are around 1.5 μm in diameter, as are RCBs. Mature chloroplasts in leaf mesophyll cells are 5–10 μm across, which might exceed the size capacity of starvation-induced autophagosomes. For instance, chloroplasts found within the vacuole of individually darkened leaves are 2–4 μm [70].

The colorless plastids of non-photosynthetic tissues such as roots and dark-grown hypocotyls can be visualized by plastid stromal-targeted GFP [82,98]. Plastids found in dark-grown hypocotyls of Arabidopsis seedlings are 2–4 μm, and thus are smaller than mature chloroplasts but similar in diameter to vacuole-transferred chloroplasts. However, RCB-type piecemeal autophagy of plastids seems to occur preferentially in this tissue under sucrose-starved conditions, indicating that RCB-based autophagy must exist in response to factors other than merely size limitation of autophagy (Fig. 3; Supplemental video 1).

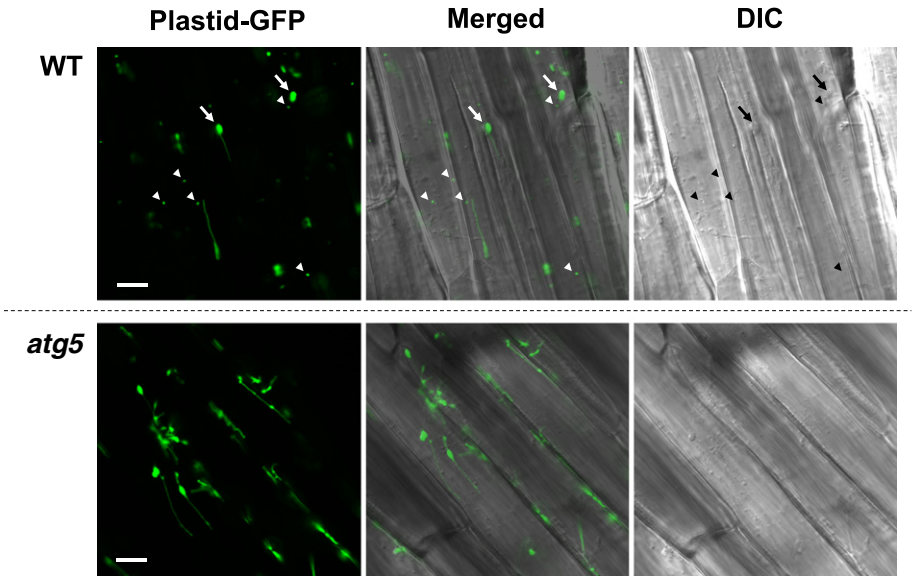

Fig. 3. Evidence for RCB-type piecemeal autophagy of plastids in dark-grown hypocotyls of Arabidopsis. Transgenic seeds for lines expressing plastid stroma-targeted GFP in wild-type (WT) and *atg5* backgrounds were grown for 5 days in darkness on agar plates containing half-strength Murashige and Skoog medium (MS) and 2% sucrose and the resulting seedlings were further incubated in liquid MS without sucrose in the presence of 1 μM concanamycin A for 1 day. Hypocotyls of seedlings were observed by laser scanning confocal microscopy. GFP fluorescence is pseudo-colored green and differential interference contrast (DIC) images of the cells and the merged images are also shown. In WT, the arrows indicate plastids that are in the plane of focus and arrowheads indicate RCB-type autophagic bodies in the vacuole. RCB-type autophagic bodies are not found in the *atg5* mutants. Bars = 10 μm.

5.3. Autophagic adaptors required for selective autophagy

In selective autophagy, autophagic adapters (cargo receptors) are responsible for recognition of specific substrates [99,100]. These adapters include Atg19 in the Cvt pathway [101], p62 and neighbor of BRCA1 gene 1 (NBR1) in degradation of ubiquitinated protein aggregates [102], and Atg32 and its mammalian homolog, Nix, in mitophagy [103–105]. The autophagic adapters interact directly with the autophagosomal marker protein Atg8 (LC3 in mammals) through a consensus sequence termed the Atg8-family interacting motif (AIM) (LC3-interacting region in mammals; LIR), and this interaction is important for the recruitment of specific substrates to autophagosomes.

Plants also have AIM/LIR-retaining autophagic adapters. Recent studies have identified plant homologues of NBR1 in Arabidopsis and tobacco [106,107]. NBR1-mediated autophagy also targets ubiquitinated protein aggregates in Arabidopsis [108]. Other plant AIM proteins have been identified, including a tryptophan-rich sensory protein (TSPO)-related membrane protein [109] and Arabidopsis Atg8-interacting proteins (referred to as ATI1 and ATI2) [110]. It is of interest to determine whether plant-specific autophagic adapter functions are related to the RCB pathway and chlorophagy.

6. Roles of chloroplast-targeted autophagy

6.1. Nitrogen remobilization during leaf senescence

The amounts of major photosynthetic proteins decrease during senescence in *atg* mutants as well as in wild-type [70,111,112]. The autophagic contribution to overall Rubisco degradation has been recently estimated using vacuolar processing assays of Rubisco-GFP fusion proteins to be at least ~40% during dark-promoted senescence [113]. Similar processing assays using Rubisco-RFP, which is resistant to vacuolar proteases under illumination, indicate that autophagy also makes a substantial contribution to degradation during natural leaf senescence [113].

A recent physiological study on whole plants showed the importance of autophagy in vegetative growth, seed production, and nitrogen remobilization in Arabidopsis [114]. Nitrogen remobilization efficiency (NRE) is lower in Arabidopsis *atg* mutants, which show premature (early) leaf senescence due to activation of salicylic acid signaling [53] that also may affect NRE. Double mutants of *atg5* NahG or *atg5 sid2*, in which salicylic acid signaling is dampened [53], show normal leaf senescence and partially recovered vegetative and seed biomass [114]. However, the NRE defect is not recovered in those double mutants. From this, the authors concluded that the autophagy machinery is in itself the main factor affecting nitrogen remobilization in *atg* mutants.

6.2. Energy metabolism in a diurnal cycle

Many plants store a portion of their photoassimilate as starch granules in chloroplasts during the day and remobilize it to support metabolism and growth at night [115]. In Arabidopsis, this leaf starch is degraded into mainly maltose and glucose within chloroplasts and then those sugars are exported to the cytoplasm across the chloroplast envelope. Starch breakdown is facilitated by glucan phosphorylation mediated by the sequential actions of glucan, water dikinase (STARCH EXCESS 1) [116] and phosphoglucan, water dikinase [117]. This disrupts the semi-crystalline packing of glucans at the granule surface, helping hydrolyzing enzymes such as β-amylases and debranching enzymes to gain access [115].

Starch granules also accumulate for storage of carbohydrates in seeds and are degraded and used for seedling growth during germination. In cotyledon cells of *Vigna mungo* seedlings, protein storage vacuoles are converted into lytic vacuoles that contain α-amylase, which can directly degrade starch [118]. These lytic vacuoles take up starch granules through autophagy in a manner that is morphologically similar to yeast micropexophagy [118].

A recent study showed autophagy can contribute to leaf transitory starch degradation. Massive starch accumulation is observed in *ATG6*-silenced *Nicotiana benthamiana* and in Arabidopsis *atg* mutants [80]. In particular, the Arabidopsis *atg5* mutant accumulates more than 8 times as much starch as wild-type in soil culture under long-day conditions [80]. Microscopic observations suggest that small starch granule-like structures, around 0.5 μm in diameter, are sequestered by autophagosomes and delivered to the vacuole by RCB-type piecemeal autophagy [80]. A co-silencing assay of intra-plastidic pathways for starch degradation (*STARCH EXCESS 1*) and autophagy (*ATG6*) suggested that the pathways independently contribute to leaf starch degradation

[80]. However, around the same time, two other studies showed that leaf starch turnover is normal in the Arabidopsis *atg5* mutants [111,119]. The underlying reasons for these reported contradiction differences in regarding starch accumulation in *atg5* mutants are currently unknown, and further detailed studies are needed to evaluate how much autophagy contributes to leaf starch degradation.

Alternatively, autophagy can contribute to energy supply at night, possibly by supplying alternative respiration substrates such as amino acids [119]. RCB autophagy is active when sugar availability in leaves is limited [71]. During a diurnal cycle, RCBs are highly accumulated in leaves excised at the end of the night with low starch content, but are less abundant in leaves at the end of the day with high starch content. Starchless mutants, such as *phosphoglucomutase* (*pgm*) [120] and *ADP-Glc pyrophosphorylase1* (*adg1*) [121], produce a large number of RCBs during incubation in darkness. These results suggest a role for RCB/autophagy in energy production via degradation of chloroplastic proteins when photosynthetic carbon assimilation is restricted.

Arabidopsis *atg* mutants show reduced growth particularly under short-day growth conditions [119]. The growth retardation of *atg* mutants is relieved under continuous light or by feeding of exogenous sucrose under short-day conditions. Starchless mutants also show short-day dependent growth retardation [120,121]. The phenotypes of *atg* and starchless double mutants are additive and more severe than those in single mutants; their growth almost ceases and their leaves show early cell death under short days [71]. Transcript analysis of dark-inducible genes indicates that the sugar starvation symptoms observed in starchless mutants become more severe in starchless *atg* double mutants, supporting the involvement of autophagy in maintaining the plant's energy supply [71].

In plants, respiration is primarily dependent on sugar oxidation. However, plants use alternative respiratory substrates such as proteins, lipids, and chlorophylls when sugar availability is limited. Recent studies have shown that the mitochondrial electron-transfer flavoprotein (ETF)/ETF:ubiquinone oxidoreductase (ETFQO) complex is induced by darkness and that it functions to transfer electrons to the ubiquinone pools in order to support respiration in Arabidopsis [122,123]. Isovaleryl-CoA dehydrogenase (IVDH) and 2-hydroxyglutarate dehydrogenase (D2HGDH) have been identified as enzymes for amino acid catabolism and electron donors to the ETF/ETFQO complex [124]. Those systems are important for catabolism of branched-chain amino acids, aromatic amino acids, and lysine [122–124]. The contents of free amino acids increase in starchless mutant leaves. However, the increases in branched chain amino acids and aromatic amino acids are partially compromised in starchless *atg* double mutants, suggesting that autophagy supplies amino acids for the ETF/ETFQO complex under carbon-limited conditions [119]. Further studies are required to examine the link between autophagy and amino acid catabolism for respiration in detail.

6.3. Adaptations to changing environments

As previously discussed [69,119], RCB-type autophagy of chloroplasts/plastids could allow a starving plant to recycle materials from proteins without destroying whole organelles. If environmental conditions subsequently improve, then chloroplasts/plastids that have undergone only loss of some protein content could be rejuvenated and resume normal function. Furthermore, when only a portion of the organelle content is removed by autophagy, some basal functions of the organelle could be maintained under starvation conditions.

In Arabidopsis roots, amyloplasts in columella cells are rapidly degraded during the hydrotropic response, possibly by both piecemeal and whole-organelle autophagy [125]. Seedling roots display not only gravitropism but also hydrotropism, and the two tropisms interfere with one another [126]. Degradation of amyloplasts involved in gravisensing enhances the hydrotropic response by reducing the gravitropic response [126]. After root tips reach the water-filled region during the hydrotropic response, columella cells regain starch-filled amyloplasts within several hours [125]. Therefore, in this case, piecemeal autophagy contributes to prompt environmental responses of plants.

6.4. Quality control of chloroplasts and stromal proteins

Reactive oxygen species (ROS) are highly reactive and can cause damage to various biomolecules, leading to cell death. Chloroplasts are one of the major sites of ROS production in plants. In illuminated chloroplasts, in which absorbed light energy is converted into chemical energy, production of reactive oxygen species is unavoidable and is enhanced under light and oxidative stress conditions [127]. Autophagy can remove proteins damaged by ROS during oxidative stress conditions in Arabidopsis [54]. In carotenoid-deficient Chlamydomonas mutants, it has recently been revealed that the absence of photoprotection leads to increased levels of ROS in the chloroplast and a pronounced increase in autophagic activity [128].

Stromal proteins such as Rubisco and Gln synthetase are susceptible to damage by ROS under oxidative conditions in chloroplasts and are directly fragmented or partially degraded by chloroplast proteases [129–133]. As previously described [32,111,114], it is possible that such damaged proteins associate with the chloroplast envelope [134] and are preferentially incorporated into RCBs. Fragments of the large subunit of Rubisco (RbcL) and Gln synthase are specifically observed in senescing leaves of several Arabidopsis *atg* mutants [111]. The occurrence of starvation-independent disposal of abnormal plastids and chloroplasts in *ppi40* and *mex1* mutants also suggests that autophagy could play a role in quality control at the whole organelle level under oxidative stress conditions. Accumulation of damaged proteins and chloroplasts may impair photosynthesis, which might partly explain the fact that autophagy mutants show lower carbon: nitrogen ratios than wild type [111].

7. Conclusions and outlooks

It is now evident that autophagy plays a central role in nutrient recycling in plants as it does in other eukaryotes. Chloroplasts are primary energy suppliers via photosynthesis, and also represent the most abundant source of nutrients and energy during senescence and under suboptimal conditions. They are transported to the central vacuole for degradation by autophagy in two distinct forms, RCBs and whole organelles. Moreover, there is an *ATG*-independent novel route by which chloroplast proteins are transferred to SAVs. This diversity of chloroplast degradation pathways may be important for both coordination of efficient recycling and maintaining the function of the organelle for plant survival in ever-changing environments. To date there are no reports comparing RCBs with SAVs in the same experimental system. This is needed in order to understand how these various pathways contribute to chloroplast degradation in plants. In parallel, a required task for the future is the identification of molecules such as cargo receptors that are involved in conferring selectivity to chloroplast-targeted autophagy.

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