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Title: Autophagy plays a role in chloroplast degradation during senescence in individually darkened leaves

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ABSTRACT
Chloroplasts contain approximately 80% of total leaf nitrogen and represent a major source of recycled nitrogen during leaf senescence. While bulk degradation of the cytosol and organelles in plants is mediated by autophagy, its role in chloroplast catabolism is largely unknown. We investigated the effects of autophagy disruption on the number and the size of chloroplasts during senescence. When leaves were individually darkened, senescence was promoted similarly in both wild-type Arabidopsis (Arabidopsis thaliana) and in an autophagy defective mutant, atg4a4b-1. The number and size of chloroplasts decreased in darkened leaves of wild-type, while the number remained constant and the size decrease was suppressed in atg4a4b-1. When leaves of transgenic plants expressing stromal-targeted DsRed were individually darkened, a large accumulation of fluorescence in the vacuolar lumen was observed. Chloroplasts exhibiting chlorophyll fluorescence as well as Rubisco-containing bodies were also observed in the vacuole. No accumulation of stroma-targeted DsRed, chloroplasts, or Rubisco-containing bodies was observed in the vacuoles of the autophagy-deficient mutant. We have succeeded in demonstrating chloroplast autophagy in living cells and provide direct evidence of chloroplast transportation into the vacuole.
INTRODUCTION

Chloroplasts contain 75-80% of total leaf nitrogen mainly as proteins (Makino and Osmond, 1991). During leaf senescence, chloroplast proteins are gradually degraded as a major source of nitrogen for new growth (Wittenbach, 1978; Friedrich et al., 1980; Mae et al., 1984), correlating with a decline in photosynthetic activity, while chloroplasts gradually shrink and transform into gerontoplasts, characterized by the disintegration of the thylakoid membranes and accumulation of plastoglobuli (for a recent review, see Krupinska, 2006). Concomitantly, a decline in the cellular population of chloroplasts is also evident in many cases, for example during natural (Kura-Hotta et al., 1990; Inada et al., 1998), dark-induced (Wittenbach et al. 1982) and nutrient-limited senescence (Mae et al., 1984; Ono et al. 1995), suggesting the existence of a whole chloroplast degradation system. Some electron-microscopic studies have shown whole chloroplasts in the central vacuole, which is rich in lytic hydrolases (Wittenbach et al., 1982; Minamikawa et al., 2001). However, there is no direct evidence of chloroplasts moving into the vacuole in living cells, and the mechanism of transport is not yet understood (Hörtensteiner and Feller, 2002; Krupinska, 2006).

The most abundant chloroplast protein is ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39), comprising approximately 50% of the soluble protein (Wittenbach, 1978). The amount of Rubisco decreases rapidly in the early phase of leaf senescence, although more slowly in the later phase (Friedrich et al., 1980; Mae et al., 1984). In contrast, the chloroplast number remains relatively constant, making it impossible to explain Rubisco loss solely by whole chloroplast degradation. However, the mechanism of intra-chloroplastic Rubisco degradation is still unknown (for a review, see Feller et al., 2008). Using immuno-electron microscopy (IEM), we previously demonstrated in naturally senescing wheat (Triticum aestivum) leaves that Rubisco is released from chloroplasts into the cytoplasm and transported to the vacuole for subsequent degradation in small spherical bodies, named Rubisco-containing bodies (RCBs; Chiba et al., 2003). Similar chloroplast-derived structures were also subsequently confirmed in senescent leaves of soybean (Glycine max) and/or Arabidopsis (Arabidopsis thaliana) by electron
microscopy (Otegui et al., 2005), and recently in tobacco (Nicotiana tabacum) leaves by IEM although the authors gave them a different name, Rubisco-vesicular bodies (RVBs; Prins et al., 2008). RCBs have double membranes which seem to be derived from the chloroplast envelope, thus, the RCB-mediated degradation of stromal proteins represents a potential mechanism for chloroplast shrinkage during senescence. We recently demonstrated that Rubisco and stromal-targeted fluorescent proteins can be mobilized to the vacuole by ATG-dependent autophagy via RCBs, using leaves treated with concanamycin A, a vacuole H^+-ATPase inhibitor (Ishida et al., 2008). To investigate further, we wished to observe chloroplast autophagy and degradation directly in living cells to determine whether autophagy is responsible for chloroplast shrinkage and whether it is involved in the vacuolar degradation of whole chloroplasts during leaf senescence.

Autophagy is known to be a major system for the bulk degradation of intracellular proteins and organelles in the vacuole in yeast and plants, or the lysosome in animals (for detailed mechanisms, see reviews, Ohsumi, 2001; Levine and Klionsky, 2004; Thompson and Vierstra, 2005; Bassham et al., 2006). In those systems, a portion of the cytoplasm, including entire organelles, is engulfed in membrane-bound vesicles and delivered to the vacuole/lysosome. A recent genome-wide search confirmed that Arabidopsis has many genes homologous to the yeast autophagy genes (ATGs; Hanaoka et al., 2002; Doelling et al., 2002; for detailed functions of ATGs, see the reviews noted above). Using knockout mutants of ATGs and a monitoring system with an autophagy marker, GFP-ATG8, numerous studies have demonstrated the presence of the autophagy system in plants and its importance in several biological processes (Yoshimoto et al., 2004, Thompson et al., 2005; Suzuki et al., 2005; Liu et al, 2005; Xiong et al., 2005, 2007, Fujiki et al., 2007, Phillips et al., 2008). These papers suggest that autophagy plays an important role in nutrient recycling during senescence, especially in nutrient starved plants. The atg mutants exhibited an accelerated loss of some chloroplast proteins, but not all, under nutrient-starved conditions and during senescence, suggesting that autophagy is not the sole mechanism for the degradation of chloroplast proteins; other as yet unidentified systems must be responsible for the degradation of chloroplast contents when the ATG system is compromised (Levine
and Klionsky, 2004; Bassham et al., 2006). However, it still remains likely that autophagy is responsible for the vacuolar degradation of chloroplasts in wild-type plants.

Prolonged observation is generally required to follow leaf senescence events in naturally aging leaves, and senescence associated processes tend to become chaotic over time. In order to observe chloroplast degradation over a short period, and to draw clear conclusions, a suitable experimental model of leaf senescence is required. Weaver and Amasino (2001) reported that senescence is rapidly induced in individually darkened leaves (IDLs) of Arabidopsis, but retarded in plants subjected to full darkness. In addition, Keech et al. (2007) observed a significant decrease of both the number and the size of chloroplasts in IDLs within six days.

In this study, using IDLs as a senescence model, we aimed to investigate the involvement of autophagy in chloroplast degradation. We show direct evidence for the transport of whole chloroplasts and RCBs to the vacuole by autophagy.

RESULTS

Senescence induced in IDLs is normal in both wild-type and atg mutant Arabidopsis

To examine the role of autophagy in chloroplast degradation, we chose a T-DNA insertion double mutant of ATG4, atg4a4b-1, which completely lacks autophagy (Yoshimoto et al., 2004). We first observed senescence in IDLs of wild-type and atg4a4b-1. Dark treatment was applied to the expanding third and fourth rosette leaves (Fig. 1A, Day 0) when both wild-type and atg4a4b-1 plants started bolting under long day conditions. During the treatment, there were no apparent differences between wild-type and atg4a4b-1 in the control condition or in the unshaded leaves of the IDL-treated plants (Fig. 1A, Day 3). In both plants, darkened leaves became pale after 5-d treatment (Fig. 1A, Day 5). SAG12 and SEN1 are well-known senescence markers (Weaver et al., 1998). In IDLs, expressions of both genes were accelerated. Over the course of leaf senescence, photosynthesis-related nuclear genes were preferentially down-regulated. The expression of RBCS2B, coding for
the Rubisco small subunit, and \textit{CAB2B}, coding for the light-harvesting chlorophyll a/b binding protein, was suppressed in IDLs more rapidly than under control condition in both plants.

In both wild type and \textit{atg4a4b-1}, a decrease in the amount of chlorophyll in IDLs was readily observable (Fig. 1A), as compared to control conditions (Fig. 2). Under control conditions, leaf nitrogen, soluble protein and Rubisco protein concentrations slowly declined in both wild-type and the mutant lines (Fig. 2). By contrast, IDLs exhibited much higher rates of loss of them in both plants (Fig. 2). These data confirmed that senescence is rapidly induced in IDLs within short periods under our experimental conditions, similar to previous studies (Weaver and Amasino, 2001; Keech et al., 2007).

\textbf{The number and the size of chloroplasts decreased in wild-type Arabidopsis, but not in the \textit{atg} mutants}

In IDLs of wild-type, all \textit{ATGs} transcripts analyzed were steadily upregulated during the treatment (Fig. 3), suggesting that autophagy may be responsible for the noted degradative processes during senescence. In \textit{atg4a4b-1}, the expression of \textit{ATGs}, 5, 7, and 9, were still up-regulated (Fig. 3), although the processing reaction of \textit{ATG8} was compromised and the progression of autophagy was completely prevented (Yoshimoto et al., 2004).

Changes in the number and the area of chloroplasts were measured during the 5-d treatment in both wild-type and \textit{atg4a4b-1} (Fig. 4). After 5-d under control conditions, chloroplasts still retained their ellipsoid shape and no apparent differences were evident between wild-type and \textit{atg4a4b-1} cells (Fig. 4A). By contrast, the occupancy of chloroplasts in the cell apparently decreased in IDLs, with the reduction more severe in wild-type cells than \textit{atg4a4b-1} cells (Fig. 4A). After 5-d IDL treatment, chloroplasts had a round shape in wild-type cells (Fig. 4A). The chloroplast number gradually increased in both plants under control conditions (Fig. 4B), suggesting that mesophyll cells were still slightly expanding during the experimental period. Conversely, in the IDL treatment, chloroplast number significantly decreased in wild-type cells, while remaining constant in
*atg4a4b-1* (Fig. 4B). Chloroplast area remained constant in both plants under control conditions (Fig. 4C), but gradually decreased in wild-type plants in the IDL treatment (Fig. 4C). After 5-d treatment, the wild-type chloroplasts had shrunk by one-third. However, in the IDLs of *atg4a4b-1*, the area decreased after 1-d and remained constant thereafter (Fig. 4C). These results strongly suggest that autophagy is responsible for the decrease in chloroplast number and partly for the decrease in chloroplast size.

**Direct evidence for the mobilization of whole chloroplasts and RCBs to the vacuole for autophagy in IDLs**

To directly demonstrate the autophagy-dependent transport of whole chloroplasts and RCBs to the vacuole in IDLs, we first observed the fate of chloroplast components in living mesophyll cells by laser-scanning confocal microscopy (LSCM; Fig.5), using transgenic Arabidopsis expressing chloroplast stromal-targeted DsRed (CT-DsRed; Ishida et al. 2008). As previously reported, DsRed fluorescence was detected within chloroplasts, but not in the vacuole of mesophyll cells when excised leaves of these plants grown under control condition were immediately observed (Ishida et al., 2008; Fig. 5A). By contrast, a faint signal of DsRed fluorescence was observed in the vacuolar lumen in mesophyll cells of IDLs (Fig. 5B). When the control leaves and IDLs of CT-DsRed-containing plants were analyzed by immunoblotting with anti-DsRed antibodies following SDS-PAGE, a single band of 28.3-kD, which corresponds to the mature form of CT-DsRed after cleavage of the transit sequence, was detected in both leaves (Supplemental Fig. S1). On the other hand, a 34.0-kD band that would correspond to the pre-mature form of DsRed carrying the RECA-transit peptide was not found, even upon overexposure of the immunoblot, suggesting that DsRed fluorescence found in the vacuole of IDLs reflects the mobilization of the stromal-localized proteins to the vacuole.

A few spherical DsRed fluorescent bodies, namely RCBs (Ishida et al., 2008), were also observed in the vacuolar lumen of IDLs (Fig. 5B, arrowhead). Detection of RCBs increased during the IDL treatment (Table 1). In addition, some cells in IDLs after 3- and 5-d contained chloroplasts without DsRed fluorescence (Fig. 5B, arrow; Table 1). While
chloroplasts are normally observed around the cell periphery, the noted chloroplasts exhibited Brownian motion, suggesting a vacuolar location, along with the RCBs. To further confirm the location of chloroplasts without DsRed fluorescence within cells, vacuoles were isolated from IDLs. DsRed-free chloroplasts were found within some of the isolated vacuoles exhibiting slow random motion (Fig. 6; Supplemental Video S1). DsRed-free chloroplasts in the vacuole were 3 to 4 μm in diameter, slightly smaller than chloroplasts in the cytoplasm, but rather bigger than RCBs, which were approximately 1 μm in diameter. When the fluorescence spectra of these chloroplasts in the vacuole and RCBs were investigated in living cells of IDLs (Fig. 7), RCBs exhibited the same fluorescence spectrum as chlorophyll-free amyloplasts in the roots of the transgenic Arabidopsis expressing plastid-targeted DsRed. While, chloroplasts in the vacuole had no fluorescent peak corresponded to DsRed but showed the specific chlorophyll autofluorescence spectrum. These data suggest that RCBs and chloroplasts in the vacuole are clearly distinguishable. Concanamycin A, an inhibitor of vacuolar H^+-ATPase, is used to suppress vacuolar lytic activity by increasing the interior pH of the vacuole, and to visualize autophagic bodies (Yoshimoto et al., 2004; Thompson et al., 2005). RCBs have also been identified in living cells in the presence of concanamycin A (Ishida et al., 2008). In light of these reports, IDLs were excised and treated with concanamycin A to monitor accumulated substances in the vacuole more clearly. After the treatment, stroma-targeted DsRed fluorescence was clearly observable in the vacuolar lumen (Fig. 5C), and both RCBs and chloroplasts were visible in higher numbers in the presence of concanamycin A after the treatment (Fig. 5B,C; Table 1).

In contrast to the results in wild-type plants, DsRed fluorescence was not observed in the vacuoles of IDLs or control condition leaves excised from CT-DsRed transgenic atg4a4b-1 plants (Table 1; Supplemental Fig. S2A,C). Even after concanamycin A treatment, accumulation of RCBs and chloroplasts exhibiting chlorophyll autofluorescence in the vacuole was not observed in atg4a4b-1 in either the leaves of control condition or IDLs (Table 1, Supplemental Fig. S2B,D).
DISCUSSION

Although pioneer studies suggested the vacuolar uptake of chloroplasts during leaf senescence (Wittenbach et al., 1982; Minamikawa et al., 2001), direct evidence and the underpinning mechanism(s) have remained elusive. In addition, recent reverse-genetic studies have revealed that autophagy is responsible for the vacuolar degradation of cellular components in plants (Yoshimoto et al., 2004; Thompson et al., 2005; Suzuki et al., 2005; Xiong et al., 2005, 2007; Phillips et al., 2008). In this study, we showed that the *atg4s* mutation, which compromises the progression of autophagy, prevents the decrease in chloroplast number and in part of the decrease in chloroplast size during IDL senescence. We also provide direct evidence of chloroplasts as well as their derivative structures, RCBs, being taken up by the vacuole in an autophagy-dependent manner using live cell imaging and cellular fractionation.

In IDLs of *atg4a4b-1*, after an initial decrease in chloroplast area, the area remained constant over the treatment period, whereas it significantly decreased in IDLs of wild-type plants (Fig. 4C). Rubisco concentration declined similarly in wild-type and *atg4a4b-1* plants during entire IDL treatment period (Fig. 2), suggesting that chloroplast shrinkage does not necessarily correlate with the loss of protein. RCBs accumulate in the vacuole of IDLs in wild-type plants but not at all in *atg4a4b-1*, suggesting that the derivation of RCBs from chloroplasts is partly responsible for their shrinkage. Chloroplasts in IDLs of *atg4a4b-1* were a jagged ellipsoid shape whereas they were smooth and round in IDLs of wild-type plants (Fig. 4A). A previous EM study indicated that RCBs have double membranes which are derived from the chloroplast envelope (Chiba et al., 2003). These observations indicate that the RCB formation is accompanied by the loss of chloroplast envelope resulting in chloroplast shrinkage. However, despite being unable to form RCBs the chloroplast area decreased by approximately 70% in *atg4a4b-1* within 1-d after the darkness was imposed. Although the specific cause for this decrease in chloroplast area is unknown, it may be related to the degradation of starch granules which occupy a large volume in the chloroplasts. Starch content rapidly decreases when leaves or plants are
placed in darkness, even in isolated chloroplasts (Stitt and Heldt, 1981).

During IDL-induced senescence, in spite of the lack of autophagy in \textit{atg4a4b-1}, the amounts of Rubisco protein and leaf nitrogen decreased at almost the same rate as control plants (Fig. 2). This suggests the presence of multiple protein degradation pathways, and that such pathway(s) are enhanced when autophagy is defective. This may explain the increased rate of chlorophyll loss in \textit{atg} mutants compared with wild-type plants (Fig. 2). This suggests that the thylakoid membranes are degraded faster in \textit{atg4a4b-1} than in wild-type. However, the loss of chlorophyll did not correlate with the chloroplast shrinkage (Figs. 2 and 4), the reason for which are unknown. Rubisco content generally decreases at a faster rate than chlorophyll content during senescence. Therefore, it is possible that a significant perturbation in senescence progression occurs when autophagy is defective, because of the importance of autophagy for chloroplast degradation. Meanwhile, the plants subjected to full darkness ceased growth, and leaf senescence proceeded more slowly than in the IDL treatment. In the leaves of completely darkened plants, the declines in chloroplast size and number, and in Rubisco, chlorophyll and nitrogen content in both wild-type and \textit{atg4a4b-1} were reduced (data not shown). Thus, the senescence of IDLs is not caused solely by the effect of darkness, but seems to be dependent upon the nutrient requirements of developing tissues.

Compared to natural and nutrient-limited senescence, which progress gradually from the older leaves in a plant and the older parts of leaves, senescence induced by IDL treatment takes place regardless of cell age and is regulated at the cellular level. Following Weaver and Amasino (2001), using a leaf which had an IDL treatment applied using a cover with has a hole in the center, we confirmed that both visible leaf yellowing and accumulation of stroma-targeted DsRed, RCBs, and chloroplasts in the vacuole are observed only in the darkened areas of the leaf (data not shown). IDL-induced senescence, including autophagy induction, may have a physiological role in the efficient allocation of nutrients under dark conditions. IDL treatment may induce intracellular degradation more aggressively than natural aging. Therefore, IDL treatment is a useful model for observation of chloroplast degradation both in number and size in a short period, however, it is unclear
how closely IDLs senescence resembles natural senescence. For example, chloroplast shrinkage precedes the decrease in chloroplast numbers observed during natural and nutrient-limiting senescence (Ono et al., 1995; Inada et al., 1998). When natural-aging leaves were subjected to 20 h-darkness with concanamycin A, autophagy of only RCBs, not whole chloroplasts, was observed (Ishida et al., 2008). Therefore, the occurrence of autophagy of whole chloroplasts and RCBs may be dependent upon the cellular status and may be a highly regulated phenomenon for chloroplast degradation.

Recently, it has been shown that senescence-associated vacuoles (SAVs) are involved in the degradation of chloroplastic components in senescing leaves of tobacco (Martínez et al., 2008). SAVs preferentially contain stromal proteins such as Rubisco and Gln synthetase, and chlorophyll a in some cases, but do not contain thylakoid proteins. These features are similar to these for RCBs, except that RCBs do not exhibit chlorophyll autofluorescence (Fig. 7). Most importantly, SAVs are present in leaves of the atg7 mutant of Arabidopsis (Otegui et al., 2005), in which the ATG-gene dependent autophagy, and therefore RCB production, is defective (Doelling et al., 2002; Thompson et al., 2005). Therefore, it should be considered that at least two independent transporting pathways, the ATG gene-dependent autophagy to the central vacuole and ATG-independent unidentified mechanism(s) to SAVs, are responsible for the degradation of stromal proteins outside chloroplasts. At present, however, we can only rarely visualize the existence of stroma-targeted fluorescent proteins other than those located in chloroplasts, including stromules and the central vacuole in natural senescing leaves or IDLs of both wild-type and autophagy-defective Arabidopsis (Ishida et al., 2008; this study).

MATERIALS AND METHODS

Plant materials and treatment

The Arabidopsis thaliana (L) Heynh. ecotype Wassilewskija and a T-DNA knockout mutant of atg4a4b-1 with this ecotype (Yoshimoto et al., 2004) were used in this study. Transgenic Arabidopsis Wassilewskija expressing stromal-targeted DsRed was previously
described (Ishida et al., 2008). Transgenic Arabidopsis Wassilewskija with \textit{atg4a4b-1} background expressing stromal-targeted DsRed was obtained by sexual crosses. Plants were grown hydroponically on horticultural rockwool in a controlled-environment growth chamber with a 14 h photoperiod (220 \textmu mol quanta m\(^{-2}\) s\(^{-1}\)) and 22/20 °C day/night temperature.

**IDLs treatment**

The expanding third and fourth rosette leaves of 19-day-old plants were used for IDL treatments, except for vacuole isolation. IDLs treatments were conducted as previously described (Weaver and Amasino, 2001; Keech et al., 2007), except in the IDLs treatment, third and fourth rosette leaves were covered with aluminum foil for 1, 3 or 5 d.

**Semi-quantitative RT-PCR**

RNA was isolated from leaves using the RNeasy Plant Mini Kit (QIAGEN). RNA concentrations were measured spectrophotometrically and an RNA gel was run from each batch of RNA samples in order to check the quality of the RNA and the accuracy of the concentration. Total RNA was treated with DNase (DNA-free: Ambion) prior to the synthesis of first-strand cDNA by Superscript III first-strand synthesis system for RT-PCR with random hexamers primers (Invitrogen). Gene-specific primers used for senescence marker genes PCR were 5’-GATGAAGGCAGTGACACACAA-3’ and 5’-TCCCACACAAACATACACAATTAAAAGC-3’ for \textit{SAG12} (Panchuk et al., 2005), 5’-ATCAGGAATGGAAACTG G-3’ and 5’-CTTTCCCTCCATCGGAAG-3’ for \textit{SEN1} (Hanaoka et al., 2002), 5’-ACCTTCTCCGCAACAAGTGG-3’ and 5’-GAAGCTTGCTGGCTTGAGG-3’ for \textit{RBSC2B} (Acevedo-Hernández et al., 2005) and 5’-TTGAAGGCTACAGAGTCCGAGGAAA-3’ and 5’-CACTCAGGAAGCAAGACTGAAGCA-3’ for \textit{CAB2B} (Panchuk et al., 2005). Gene-specific primers used for autophagy genes PCR were 5’-ATGAAAGCTTTATGATAGATTTGTTC-3’ and 5’-TCAGAGCATTGGCCAGTCTCTTCA-3’ for \textit{ATG4a},
5’-ATGAAGGCTTTATGTGATAGATTTGTTC-3’ and 5’-GTCACACAATGAAAAGAATGGCTAGGAG-3’ for ATG4b (Yoshimoto et al., 2004), 5’-ATGGCGAAGGAAGCGGTCA-3’ and 5’-CACAAAGGAGATCGAAAAGAATGGCTAGGAG-3’ for ATG5 (Thompson et al., 2005), 5’-AGCTCTTGAAGACCCTTCTGTG-3’ and 5’-AATCTGAGTCGCGCCAATC-3’ for ATG7, 5’-GTCCCTCGACCCAGTACGGAACCTATG-3’ and 5’-AGAAACTGCTTCCAAAGAGAATCTG-3’ for ATG9 and QuantumRNA 18s Internal standards for 18s ribosomal RNA (Ambion). PCR was terminated after 28 cycles for SAG12, 23 cycles for SEN1, 20 cycles for RBCS2B, CAB2B, 14 cycles for 18s ribosomal RNA, 24 cycles for ATG4a, 4b and 26 cycles for ATG5, 7 and 9.

**Quantification of chlorophyll, leaf-N, soluble proteins and Rubisco protein**

Frozen third and fourth rosette leaves were homogenized in a chilled mortar and pestle in 50 mM Na-phosphate buffer (pH 7.5) containing 2 mM iodoacetic acid, 0.8% (v/v) 2-mercaptoethanol and 5% (v/v) glycerol (Makino et al., 1988). Chlorophyll was determined by the method of Arnon (1949). Total leaf N was determined from part of the homogenate with Nessler’s reagent after Kjeldahl digestion. Triton X-100 (0.1%, final concentration) was added to the remaining homogenate, which was then centrifuged at 15,000×g for 10 min at 4°C, before the determination of soluble proteins and Rubisco in the supernatant. Soluble proteins were measured according to Bradford (1976) using a Bio-Rad protein assay (Bio-Rad) with bovine serum albumin as the standard. The Rubisco content was also determined in the supernatant spectrophotometrically by formamide extraction of the Coomasie brilliant blue R-250-stained bands corresponding to the large and small subunits of Rubisco separated by SDS-PAGE using calibration curves made with bovine serum albumin.

**Measurement of the number and the size of chloroplasts**

Leaves were fixed by a procedure described by Pyke and Leech (1991). Fixed leaf tissues were dispersed and pressed on a glass slide. Individually separated cells were
observed by laser-scanning confocal microscopy (LSCM) performed with a Nikon C1si system equipped with a CFI Plan Apo VC60× water immersion objective (NA=1.20; Nikon). Chloroplasts were identified by chlorophyll autofluorescence and the number and the size were measured in differential interference contrast images. Chloroplast number was counted with changing focus to avoid duplicate and uncounted chloroplasts. Measurements of chloroplast length and breadth were taken only on chloroplasts in the center of the cell, while chloroplast areas were calculated with the assumption that chloroplasts were oval.

Image analysis with LSCM

LSCM was performed with a Nikon C1si system equipped with a CFI Plan Apo VC60× water immersion objective (NA = 1.20; Nikon). Leaves were mounted on slides with cover slips. DsRed was excited with the combination of the 488 nm line of a multi-Argon ion laser and the 543 nm line of a Helium-Neon Laser. Chlorophyll was excited with the 488 nm line of a multi-Argon ion laser. The emission of DsRed and chlorophyll was detected between 590 and 650 nm, and over 650 nm by a multi channel detector with filters. For obtaining fluorescence spectra of RCBs and chloroplasts in the vacuole, DsRed and chlorophyll were excited using the above method, and fluorescence spectra between 550 and 710 nm were obtained with a 5-nm resolution using the spectral detector of the Nikon C1si system. The concanamycin A treatment was applied with the procedure described in Ishida et al. (2008).

Isolation of vacuoles from IDLs

Vacuoles were isolated by the method of Robert et al. (2007), with a slight modification that applied gradient fractionation with 0, 6 and 10% Ficoll solution.

Supplemental Data

Supplemental Figure S1. A, Diagram of the gene construct of stromal-targeted DsRed.
35S35S, double cauliflower mosaic virus 35S promoter; CT, transit sequence of Arabidopsis RECA gene. Molecular weight of mature DsRed is 26.3-kD, after transport into the stroma and cleavage of the transit peptide (CT). B, Immunoblot analysis of stroma-targeted DsRed in wild-type plants with monoclonal anti-DsRed antibody. Control leaves before (lane 1) or after the 5-d treatment (lane 2) and IDLs after the 5-d treatment (lane 3) were homogenized, and proteins extracted from equal leaf areas, and the equivalent to 20 µg protein in the case of lane 1, was applied to immunoblotting following SDS-PAGE. 28.3-kD bands were found in each lane while a 34-kD band, which corresponds to an unprocessed form of CT-DsRed, was not detected in any lanes.

Supplemental Figure S2. Visualization of stroma-targeted DsRed and chlorophyll autofluorescence in living mesophyll cells of atg4a4b-1. A and C, fresh leaves excised from the control plants (A) and IDLs (C) after the 5-d treatment were observed immediately. B and D, excised control leaves (B) and IDLs (D) were incubated with 1 µM concanamycin A in 10 mM MES-NaOH (pH 5.5) at 23°C for 20 h in darkness. Stromal-targeted DsRed appears green and chlorophyll fluorescence appears red. In merged images, overlap of DsRed and chlorophyll fluorescence appears yellow. Bars = 25 µm.

Supplemental video S1. Movement of chloroplasts lacking CT-DsRed in a vacuole isolated from IDLs of wild-type plants. The movie runs at 3 times normal speed.

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FIGURE LEGENDS

**Figure 1.** Induction of senescence in individually darkened leaves (IDLs) of wild-type and atg4a4b-1 plants. A, Photographs of wild-type and atg4a4b-1 plants before (Day 0) and after the 3-d treatment (Day 3) and their control leaves and IDLs after the 5-d treatment (Day 5). The third and fourth rosette leaves in order of appearance, (indicated on the day 0 plants), were individually covered just after bolting and the plants grown for a further 5 d. B, Expression of SAG12, SEN1, RBCS2B, and CAB2B in control leaves and IDLs of wild-type and atg4a4b-1 plants during the 5-d treatment. Total RNA from third and fourth leaves of each plant was isolated and subjected to semiquantitative RT-PCR using gene-specific primers. 18s rRNA was used as an internal standard.

**Figure 2.** Changes in the chlorophyll, nitrogen, soluble protein, and Rubisco protein concentrations in control leaves and IDLs of wild-type (open circles) and atg4a4b-1 (closed circles) over the treatment period. Data are means ± SD (n = 3).

**Figure 3.** Expression of ATGs in control leaves and IDLs of wild-type and atg4a4b-1 plants over the treatment period. Total RNA from third and fourth leaves of each plant was isolated and subjected to semiquantitative RT-PCR using gene-specific primers. 18s rRNA was used as an internal standard.
**Figure 4.** Visible chloroplast catabolism proceeds in IDLs of wild-type plants, but is suppressed in those of *atg4a4b-1*. A, Differential interference contrast images of chloroplasts in mesophyll cells separated from leaves of wild-type (a) and *atg4a4b-1* (d) plants before the treatment, from control leaves of wild-type (b) and *atg4a4b-1* (e) plants after the 5-d treatment, and from IDLs of wild-type (c) and *atg4a4b-1* (f) plants after the 5-d treatment. Leaves were cut into small pieces, fixed with 3.5% glutaraldehyde and mesophyll cells were individually dispersed on the glass plate and observed by microscopy. Bars = 10 μm. B and C, Changes in the number (B) and the area (C) of chloroplasts in control leaves and IDLs of wild-type (open columns) and *atg4a4b-1* (closed columns) plants during the treatment. Chloroplasts in mesophyll cells were identified by those exhibiting chlorophyll autofluorescence during laser scanning confocal microscopy. The number of chloroplasts per cell and their length and breadth were counted on differential interference contrast images (shown as A) of mesophyll cells separated from leaves. Chloroplast area was calculated using the assumption that chloroplasts were ovals. Data are means ± SD (*n* = 50 in the number per cell; *n* = 45 in the area). Statistical analysis was performed by Tukey-Kramer’s HSD test. Different letters in each graph denote differences at *P* ≤ 0.01.

**Figure 5.** Visualization of stroma-targeted DsRed and chlorophyll autofluorescence in living mesophyll cells of wild-type plants by laser scanning confocal microscopy. A and B, fresh control leaves (A) and IDLs (B) excised from plants after the 5-d treatment and observed immediately. C, IDLs from plants after the 5-d treatment incubated with 1 μM concanamycin A in 10 mM MES-NaOH (pH 5.5) at 23°C for 20 h in darkness. Stromal-targeted DsRed appears green and chlorophyll fluorescence appears red. In merged images, overlap of DsRed and chlorophyll fluorescence appears yellow. Vesicles with DsRed were represented white arrowheads. Chloroplasts having only chlorophyll fluorescence were represented arrows. D and E, magnifications of vesicles and chloroplasts having only chlorophyll fluorescence indicated by the dashed-line areas in B and C, respectively. Bars = 25 μm.
Figure 6. Detection of chloroplasts exhibiting chlorophyll fluorescence in vacuoles isolated from wild-type IDLs. Vacuoles were released from the thermally lysed protoplasts, which were prepared from 5-d treated IDLs of plants expressing stroma-targeted DsRed, and were fractionated by a Ficoll density gradient. Stroma-targeted DsRed appears green, chlorophyll fluorescence appears red, and an image by differential interference contrast (DIC) is shown in gray. Some isolated vacuoles accumulated both stroma-targeted DsRed and chloroplasts shown as the overlap of DIC images and chlorophyll fluorescence. Merged images are taken from Supplemental Video S1. Bar = 10 μm.

Figure 7. Distinction between Rubisco-containing bodies (RCBs) and chloroplasts incorporated into the vacuole by fluorescence spectra. A, Visualization of RCBs and chloroplasts in the vacuole of excised IDLs by LSCM with a spectral detector. Emission between 565 and 615 nm reflecting DsRed appears pseudo-colored green, and emission between 650 and 710 nm reflecting chlorophyll fluorescence appears pseudo-colored red. The merged image is shown in which the overlap of DsRed and chlorophyll appears in yellow. Bar = 10 μm. B, Difference in fluorescence spectra of RCBs and chloroplasts in the vacuole. Spectra between 550 and 710 nm of a chloroplast in the cytoplasm (indicated by circle a in A), a chloroplast in the vacuole (circle b in A), and a RCB (circle c in A) are obtained at 5-nm resolution, and are shown as yellow squares, green triangles, and red circles, respectively. Chlorophyll fluorescence of a wild-type chloroplast (gray circles) and DsRed fluorescence of a chlorophyll-free plastid in roots of the transgenic plants (gray triangles) are shown as references. C, Difference in the ratio of peak of DsRed fluorescence (592 nm) per peak of chlorophyll fluorescence (682 nm) of RCBs and chloroplasts in the vacuole. Data are means ± SD (n = 10). Statistical analysis was performed by t-test. Different letters denote differences at P ≤ 0.01.
Table 1. Quantitative analysis of vacuolar transfers of RCBs, chloroplasts, and stroma-targeted DsRed.

| Treatment Span for treatment | RCB  | Whole Cp | Intensity of DsRed in Vacuole |
|------------------------------|------|----------|-------------------------------|
| Control 5 day                | 0    | 0        | 4.1 ± 2.6^d                  |
| IDLs 1 day                   | 21   | 0        | 20.0 ± 8.1^c                 |
| 3 day                        | 49   | 0        | 32.6 ± 13^c                  |
| 5 day                        | 66   | 0        | 68.3 ± 20^b                  |
| 5 day + conA 20 h            | 97   | 0        | 100.0 ± 37^a                 |

RCB and chloroplast columns indicate cell numbers having RCBs or chloroplasts in the vacuole per 100 cell sections observed by a laser-scanning confocal microscopy. Intensity of DsRed in the vacuole of wild-type background plants represents the relative intensity of DsRed accumulated in the vacuole as the “IDLs 5-d +conA 20h” criterion. Fluorescence intensity is measured at cell centre spots of images captured using the same laser power and detector gain. Values are means ± SD (n = 50). Different letters represent significant difference statistically analyzed by Tukey-Kramer’s HSD test (P ≤ 0.01). The correlation coefficient between treatment time and RCB observability is 0.9867.
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