Pexophagy Protects Plants from Reactive Oxygen Species-induced Damage under High-intensity Light

Kazusato Oikawa  
National Institute for Basic Biology  
https://orcid.org/0000-0002-1033-9089

Shino Goto-Yamada  
Jagiellonian University

Yasuko Hayashi  
Niigata University

Daisuke Takahashi  
Iwate University

Yoshitaka Kimori  
Fukui University of Technology

Michitaro Shibata  
RIKEN

Kohki Yoshimoto  
Meiji University

Atsushi Takemiya  
Yamaguchi University

Maki Kondo  
National Institute for Basic Biology

Akira Kato  
Niigata University

Keisuke Shimoda  
Niigata University

Haruko Ueda  
Konan University

Matsuo Uemura  
Iwate University  
https://orcid.org/0000-0003-0436-2976

Keiji Numata  
Kyoto University

Yoshinori Ohsumi  
Tokyo Institute of Technology

Ikuko Hara-Nishimura  
Konan University  
https://orcid.org/0000-0001-8814-1593

Shoji Mano
Article

**Keywords:** plant stress, light irradiation, reactive oxygen species, peroxisomes, autophagy

**DOI:** [https://doi.org/10.21203/rs.3.rs-104334/v1](https://doi.org/10.21203/rs.3.rs-104334/v1)

**License:** [This work is licensed under a Creative Commons Attribution 4.0 International License.](https://creativecommons.org/licenses/by/4.0/)

Read Full License
Abstract

Light is essential for photosynthesis, but it has the potential to elevate intracellular levels of reactive oxygen species (ROS) during photosynthesis. Photorespiration is a metabolic pathway in photosynthesis to metabolise oxidised by products from chloroplasts, and it generates a high level of ROS in peroxisomes. Since high levels of ROS are toxic, plants must manage damage from ROS. However, the cellular mechanism to elude leaf damage from ROS in peroxisomes is not fully explored. Here we show that autophagy plays a pivotal role in the selective removal of ROS-generating peroxisomes, which protects plants from oxidative damage during photosynthesis. We found that a series of peup mutants, which is a defect in autophagy degradation of peroxisomes, showed light-intensity-dependent leaf damage and excess aggregation of ROS-accumulating peroxisomes. The peroxisome aggregates were specifically engulfed by pre-autophagosomal structures and vacuolar membranes, but they were not degraded in the mutants. ATG18a-GFP and GFP-2 × FYVE, which both bind to phosphatidylinositol 3-phosphate, preferentially targeted the peroxisomal membranes and pre-autophagosomal structures near peroxisomes in ROS-accumulated cells under high-intensity light conditions. Our findings provide new information to better understand the plant stress response caused by light irradiation.

Introduction

Photosynthesis in plants converts light energy to chemical energy and is accompanied by photorespiration, which involves peroxisomes, mitochondria, and chloroplasts. Photorespiration is essential for plant survival under high-intensity light to prevent photoinhibition, which damages photosynthetic machinery owing to excess reactive oxygen species (ROS) accumulation. Thus, understanding how excess ROS is degenerated to protect plants from oxidative damage during photosynthesis under excess light is essential.

Plants have diverse mechanisms to prevent high ROS accumulation under various conditions, and the relationship between ROS and autophagy has been reported in previous studies. ROS accumulation in peroxisomes inhibits catalase (CAT) activity that detoxifies hydrogen peroxide, leading to the oxidization of peroxisomes. We have shown that oxidative peroxisomes are undegraded in autophagy-deficient mutants.

A set of autophagy (ATG) genes are included in the specific degradation of peroxisomes, namely pexophagy in yeasts and animals. ATG proteins for the initial of autophagy form pre-autophagosomal structures (PAS) on vacuolar membranes containing phosphatidylinositol 3-phosphate (PtdIns3P) adjacent to degraded peroxisomes in yeast. Subsequently, a membrane structure called the phagophore extends from the PAS to cover peroxisomes by incorporating phosphatidylethanolamine (PE) conjugated ATG8 (ATG8-PE), and then fuses to vacuoles for degradation. Most ATG proteins are highly conserved in yeasts, animals, and plants. However, it is unclear whether pexophagy in plants is the same as in yeasts and animals, because homologues of key factors for pexophagy in...
yeasts, namely PpAtg30 and ScAtg36, are absent in plants. Moreover, no direct evidence exists for selective degradation of peroxisomes by pexophagosomes in plant cells.

Here, we investigate the cell-structural mechanism for autophagy-dependent degradation of ROS-accumulated peroxisomes to capture the pexophagosome formation in Arabidopsis leaves. Furthermore, we examined the impact of the deficiency of pexophagy on the leaf damage caused by ROS accumulation, which is enhanced by the accumulation of inactive catalases in peroxisomes. Our finding indicates a massive contribution of pexophagy on protecting plants from excess light-induced oxidative damage during photosynthesis.

Results

A different pattern of peroxisome aggregation in leaf mesophyll cells between peup1/atg2 and peup4/atg7 mutants

We previously isolated peup1/atg2, peup2/atg18a, and peup4/atg7 mutants defective in pexophagy from ethylmethane sulfonate (EMS)-mutagenised GFP-PTS1 (wild type) lines. Because the responsible genes for peups were ATGs, we obtained T-DNA insertion mutants of atg2, atg5, atg7, atg18a, and atg9. The atg mutants other than atg9, in which peroxisomes were visualised by introducing GFP-PTS1, showed peroxisome aggregation. However, we here noticed that, in detail, the peroxisome-aggregation patterns were different between peup1/atg2 and peup4/atg7 at 100 µmol m$^{-2}$ s$^{-1}$ (Fig. 1a–d and Supplementary Videos 1–3). The number of peroxisomes in peup4/atg7 was higher than in peup1/atg2 (Fig. 1b), whereas the frequency (Fig. 1c) and size (Fig. 1d) of peroxisome aggregation were less in peup4/atg7 compared with those in peup1/atg2.

Unlike non-selective, starvation-induced autophagy to recycle nutrients such as carbon and nitrogen, ROS-induced autophagy in mammals selectively degrades damaged organelles. To further obtain insight into how ROS promotes pexophagy in plants under light, we first assessed plant growth of peup1/atg2 and peup4/atg7 mutants under different light intensities (50, 100, and 200 µmol m$^{-2}$ s$^{-1}$). peup1/atg2 and peup4/atg7 leaves were more damaged than wild-type leaves (Supplementary Fig. 1a). With increases in light intensity, the mutants showed reduced chlorophyll (Supplementary Fig. 1b) and photosynthetic efficiency (Supplementary Fig. 1c). Damage to peup4/atg7 leaves was more extensive than to peup1/atg2 leaves under light; therefore, we mainly focused on peup4/atg7. ROS accumulation was higher in the damaged-peup4 leaves under 100 and 200 µmol m$^{-2}$ s$^{-1}$ light, as revealed by nitroblue tetrazolium (NTB) staining (Supplementary Fig. 1d, e). Electron microscopy analyses of peup4 revealed abnormal high-density regions in the peroxisomes (Supplementary Fig. 2a, b). These dark-grey regions contained large amounts of catalase (Supplementary Fig. 2c–f) similar to those in peup1/atg2 and atg5 mutants. Immunoblot confirmed high catalase accumulation in peup4/atg7 under light (200 µmol m$^{-2}$ s$^{-1}$), largely in the pellet fraction (Supplementary Fig. 3a–d); thus, catalase was also damaged in peup4/atg7 leaf cells. In the leaves of the peup4/atg7 mutant, tubular structures from peroxisomes,
namely peroxules, were formed, suggesting that the leaves accumulated a high level of ROS (Supplementary Fig. 4a, b and Supplementary Video 4). Interestingly, we observed that chloroplasts in the peup4/atg7 mutant ingested some peroxisomes (Supplementary Fig. 4c, d), similar to chloroplast behaviour in ROS-accumulating cells. In detailed analysis with an electron microscope, we found that some peroxisomes were ingested in curved chloroplast membranes (Supplementary Fig. 4e). These results suggest that the peup4/atg7 mutant accumulates high levels of ROS because of impairment in catalase activity, resulting in plant growth inhibition.

**ATG18a preferentially targets leaf peroxisomes in light-adapted cells**

ATG18a plays a role in autophagosome formation to degrade oxidative proteins in *Arabidopsis*, implying that ATG18a targets damaged organelles. To examine whether ATG18a is required for selective pexophagy in plants, we assessed the intracellular distribution of ATG18a-GFP in wild-type plants and peup1/atg2 and peup4/atg7 mutants bearing red fluorescence protein-fused peroxisomal targeting signal 1 (RFP-PTS1) to visualise peroxisomes in these plants (Fig. 2a). The ATG18a-GFP-containing structures localised to peroxisomes, although they are rarely observed in the wild-type plants (Fig. 2a–d). We found that many cells accumulated ATG18a-GFP structures on peroxisomes in the peup1/atg2 and peup4/atg7 mutants (Fig. 2b) and found that 30-40% of the total peroxisomes, especially aggregated peroxisomes, had the ATG18a-GFP structures (Fig. 2c). Furthermore, about 80% of the ATG18a-GFP structures localised to peroxisomes in wild-type and mutant cells (Fig. 2d), suggesting that ATG18a preferentially targets the peroxisome. Immunoblot showed ATG18a-GFP and catalase in the insoluble fraction of peup1/atg2 (Supplementary Fig. 5). Most ATG18a-GFP appeared as dot structures, but a few were cup or ring structures in peup1/atg2 and peup4/atg7 mutants at 100 μmol m⁻² s⁻¹ light intensity (Fig. 2a, e–g and Supplementary Fig. 6a, c and Supplementary Table 1). We tracked single peroxisomes by time-lapse imaging, and then the average image of RFP-PTS1 and ATG18a-GFP (Fig. 2h) was generated using a morphological image processing tool. The image revealed a ring structure of ATG18a-GFP surrounding the peroxisome in peup4. Time-lapse imaging also showed that ATG18a-GFP gradually surrounded peroxisomes in wild type and peup4/atg7, but not in peup1/atg2 (Supplementary Fig. 7a–c and Supplementary Videos 5–7). Indeed, peup1/atg2 had fewer ring structures compared with peup4/atg7 (Fig. 2f). In the peup1/atg2 mutant, 60% of fluorescence from ATG18a-GFP was recovered within 60 s after photobleaching, indicating that ATG18a-GFP rapidly accumulates at the peroxisome aggregates (Supplementary Fig. 8 and Supplementary Video 8).

To examine whether ATG18a interacts with other proteins, we conducted an immunoprecipitation of ATG18a-GFP followed by protein mass spectrometry. The result shows that various proteins of chloroplasts, peroxisome, and mitochondria were co-immunoprecipitated with ATG18a-GFP in peup1/atg2 (Supplementary Fig. 9 and Supplementary Table 2). Peroxisome proteins such as catalases (CAT1, CAT2 and CAT3), heat shock protein 70s (HSP70s), and RuBisCO-related proteins were abundantly present. We obtained the number of proteins localised to each organelle from two databases (PPDB, http://ppdb.tc.cornell.edu/dbsearch/subproteome.aspx; and SUBA4, http://suba.live) and calculated the
recovery rate. The peroxisome proteins were more efficiently recovered compared with the mitochondria and chloroplast proteins (Supplementary Fig. 9b), suggesting that many ATG18a proteins directly or indirectly bind to peroxisomes or peroxisomal proteins.

**PtdIns3P accumulates on leaf peroxisomes in light-adapted cells**

*Arabidopsis* ATG18a has a PtdIns3P-binding motif similar to yeast ATG18 and ATG21, 20, 29, 30. To validate whether peroxisomes marked by ATG18a-GFP have PtdIns3P, we examined the intracellular distribution of GFP-2×FYVE, a reliable PtdIns3P-binding marker, in *peup1/atg2* and *peup4/atg7* mutants. We found that GFP-2×FYVE showed similar trends in the fluorescence pattern (Fig. 3a, e–g and Supplementary Fig. 10 and Supplementary Videos 9, 10) and frequency of peroxisome targeting (Fig. 3b, c) as ATG18a-GFP (Fig. 2a-c, e-g and Supplementary Fig. 7 and Supplementary Videos 5, 7). These findings indicate that PtdIns3P accumulates on the membranes surrounding peroxisomes and suggest that the ATG18a-GFP recognises PtdIns3P on the membrane in the accumulation of ATG18a-GFP. We further conducted a lipid-binding test for ATG18a-GFP protein in transgenic plants. We confirmed the ability of ATG18a-GFP protein to bind to PtdIns3P (Supplementary Fig. 11a). The ring structure was also noted in GFP-2×FYVE (Fig. 3e–g and Supplementary Fig. 6b, d), but at a lesser frequency than in ATG18a-GFP (Figs. 2f, 3f and Supplementary Fig. 6a, c and Supplementary Table 1) suggesting that GFP-2×FYVE mainly functions at the initial step of ring formation. About half of the dot structures of GFP-2×FYVE did not target peroxisomes (Fig. 3d and Supplementary Table 3), indicating that there are other batteries of PtdIns3P in the cell. GFP-2×FYVE was also localised to peroxisome aggregates in the *peup2/atg18a* mutant (Supplementary Fig. 12a, b), similar to the *peup1/atg2* and *peup4/atg7* mutants, suggesting that PtdIns3P accumulation on peroxisomes precedes the action of ATG18a, ATG2, and ATG7 during pexophagy.

In detailed analysis with an electron microscope, we observed the ER and autophagosome-like structures adjacent to the high-density area in peroxisomes of *peup1/atg2* mutants (Supplementary Fig. 11b, c). We further examined the localisation of GFP-2×FYVE and ATG18a-GFP in detail with immunoelectron microscopic analysis using anti-GFP antibodies (Supplementary Fig. 11b). The results show that GFP-2×FYVE and ATG18a-GFP were localised on both peroxisomes and phagophores adjacent to the peroxisomes, suggesting that ATG18a-GFP recognises PtdIns3P on the membrane of damaged peroxisomes or PAS to initiate pexophagy in plants.

**High-intensity light causes leaf damage and high levels of peroxisomal aggregation in autophagy defective mutants**

Next, we investigated the effect of high-intensity light (1000 µmol m^{-2} s^{-1}) on damage to leaves of *peup4/atg7* mutants (Fig. 4) and T-DNA insertion mutants for *atg2, atg5, atg7*, and *atg9* (Supplementary Fig. 13). *atg2, atg5*, and especially *atg7* showed leaf damage (Fig. 4a and Supplementary Fig. 13a). Remarkably, the large aggregates of peroxisomes were induced in leaf mesophyll cells of *atg2, atg5*, and *atg7*, mostly at the cell bottom (Fig. 4b and Supplementary Fig. 13b). the frequency and size of
peroxisome aggregation in \textit{atg2}, \textit{atg5}, and \textit{atg7} under high-intensity light conditions was two to three times greater than under normal light (100 µmol m\(^{-2}\) s\(^{-1}\)) (Fig. 4c, d and Supplementary Figs. 13c, 14a, b). Peroxisome aggregation in \textit{atg2} and \textit{peup4/atg7} was evident (Fig. 4b–d and Supplementary Fig. 13b, c). The accumulation of catalase was higher in the insoluble fractions of \textit{atg2}, \textit{atg5}, and \textit{peup4/atg7} (Fig. 4e, f and Supplementary Figs. 13d, 14c). We found an increase in the number of mitochondria, which gathered in peroxisome aggregation in \textit{peup4/atg7} under high-intensity light, suggesting that mitophagy was also suppressed (Supplementary Fig. 15a–c). Mitochondrial proteins serine hydroxymethyltransferase (SHMT) and cytochrome c oxidase 2 (COXII) were increased in \textit{peup4/atg7} under high-intensity light (Supplementary Fig. 15d–g). These results suggest that ATG7 plays multiple roles in the degradation of damaged mitochondria as well as peroxisomes in leaves undergoing photosynthesis.

**High-intensity light-induced peroxisome aggregates are surrounded by vacuolar membranes together with ATG18a and PtdIns3P**

To further investigate whether high-intensity light-induced large aggregates of peroxisomes are degraded by autophagy, we focused on the subcellular localisation of ATG18a-GFP and GFP-2×FYVE in 1000 µmol m\(^{-2}\) s\(^{-1}\) light-adapted leaf cells in \textit{peup1/atg2} and \textit{peup4/atg7} mutants (Fig. 5a–d). The results show that ATG18a-GFP and GFP-2×FYVE preferentially targeted the large aggregates of peroxisomes in \textit{peup1/atg2} and \textit{peup4/atg7} mutants. Especially, peroxisome aggregations in \textit{peup4/atg7} mutants were mostly enveloped by ATG18a-GFP (Fig. 5a, b and Supplementary Table 2); the frequency of these peroxisome aggregations was about 43 % in \textit{peup4/atg7}, whereas it was about 11 % in \textit{peup1/atg2} (Fig. 5c and Supplementary Table 4). The size of peroxisome aggregates enveloped by ATG18a-GFP in \textit{peup4/atg7} was about 34 µm\(^2\), which was eight times greater than that in wild type and six times than that in \textit{peup1/atg2} (Fig. 5d). In contrast, both the frequency and size of peroxisome aggregates enveloped by GFP-2×FYVE were smaller than by ATG18a-GFP in all tested lines (Fig. 5c, d). The analysis of fluorescent intensities in the aggregates confirmed that ATG18a-GFP co-localised with the large aggregates of peroxisomes (Fig. 5e).

We further investigated the relationship between vacuolar membranes and peroxisomes in wild type and \textit{peup4/atg7} using Venus-VAM3 under high-intensity light. We found that vacuolar membrane structures surrounded peroxisome aggregates in \textit{peup4/atg7} (Fig. 5f, g). The frequency of these structures was similar to that of aggregate formations with ATG18a-GFP (Fig. 5c, h and Supplementary Tables 4, 6), suggesting a similar mechanism in these two structures. The cells with these vacuolar membrane structures were three times more abundant in \textit{peup4/atg7} than in wild type (Fig. 5h). The size of the peroxisome aggregation with the vacuolar membrane was also bigger in \textit{peup4/atg7} than in wild type and \textit{peup1/atg2} (Fig. 5i). Although the number of vacuolar bulbs was more abundant in \textit{peup4/atg7} than in wild type, the ratio of bulbs surrounding peroxisomes was similar between \textit{peup4/atg7} and wild type (Supplementary Fig. 16 and Supplementary Videos 11, 12).

**High-intensity light-induced peroxisome aggregates accumulate ROS**
We investigated the accumulation of ROS in leaves under high-intensity light (1000 µmol m\(^{-2}\) s\(^{-1}\)). NBT staining showed that the leaves of both \textit{peup1/agt2} and \textit{peup4/atg7} mutants accumulated more ROS compared with wild type (Supplementary Fig. 17a, b). Next, we examined the accumulation of ROS in peroxisomes using \(2\text{-dichlorodihydrofluorescein diacetate (H}_2\text{-DCF-DA)}\) \(^{33,34}\) (Fig. 6a, b and Supplementary Fig. 17c-e). We found that some peroxisomes in wild type and about 60% of peroxisome aggregation in the mutants were specifically stained with H\(_2\)-DCF (Supplementary Fig. 17c, d). H\(_2\)-DCF fluorescence was detected inside peroxisomes in the mutants with approximately two-fold higher intensity than in wild type (Fig. 6b). The fluorescence intensity in peroxisomes was two- to three-fold higher compared with chloroplasts; this was especially prominent in the mutants (Supplementary Fig. 17e). We concluded that ROS accumulates at high levels in peroxisome aggregates in \textit{peup1/atg2} and \textit{peup4/atg7} mutants grown under high-intensity light.

These results suggest that high-intensity light induces accumulation of high levels of ROS in peroxisomes, resulting in aggregation and subsequent degradation of peroxisomes in the vacuole by pexophagy.

**Discussion**

**Autophagy preferentially degrades ROS-accumulated peroxisomes in light**

We found leaf damage in \textit{peup1/atg2} and \textit{peup4/atg7} mutants in light (Supplementary Fig. 1). Increases in light intensity increased leaf damage, suggesting the involvement of photosynthesis. Because high-intensity light induces ROS accumulation from photosynthesis, we speculated that light-induced ROS accumulation caused leaf damage in the mutants. Indeed, we found light-dependent ROS accumulation in the leaves of \textit{peup1/atg2} and \textit{peup4/atg7} mutants, indicating that these mutants generate higher levels of ROS compared with wild type under high-intensity light. These results suggest that high levels of ROS in \textit{peup4/atg7} induce the formation of peroxules and stromules (Supplementary Fig. 4) \(^{25,26}\).

Autophagy is required for the degradation of damaged and toxic materials generated by ROS accumulation during oxidative stress \(^{13}\). However, the primary origin of ROS in leaf mesophyll cells of the autophagy-deficient mutants is still unclear. We hypothesised that the undegraded peroxisomes would primarily produce ROS in the mutants during metabolism in photorespiration. Indeed, hydrogen peroxide accumulation is higher in peroxisomes than in chloroplasts and mitochondria during photorespiration \(^{35}\). The H\(_2\)-DCF-stained aggregates of peroxisomes in the mutants confirmed the accumulation of ROS in degrading peroxisomes (Fig. 6a, b and Supplementary Fig. 17c, d). Hydrogen peroxide in peroxisomes is immediately degraded by catalase in wild-type plants. However, catalase is gradually inactivated by increasing levels of ROS in photosynthetic tissues under high-intensity light conditions. The inactivation of catalase causes over-accumulation of ROS in peroxisomes and then induces the imbalance of ROS homeostasis in cells, leading to damage and defective plant growth in the mutants \(^{4-6}\). Peroxisome participates in photorespiration through physical interaction with chloroplast and mitochondrion \(^{36}\).
Therefore, damaged peroxisome with high ROS levels should be immediately removed by pexophagy to maintain efficient metabolite flow among these organelles during photorespiration under high-intensity light conditions.

We focused on ATG18a, which is involved in the degradation of oxidative proteins, to assess how autophagy degrades peroxisomes. Because ATG18a has a well-conserved-PtdIns3P-binding domain in yeast, plant, and animals, we used GFP-2×FYVE to monitor PtdIns3P in the cell. Both GFP-2×FYVE and ATG18a-GFP preferred to target peroxisomal aggregation in peup1/atg2 and peup4/atg7 under normal light (100 µmol m⁻² s⁻¹) (Figs. 2 and 3). Furthermore, we showed that high-intensity light (1000 µmol m⁻² s⁻¹) increased the frequency and the size of peroxisome aggregates in peup1/atg2, atg5, and peup4/atg7 mutants (Fig. 4b-d and Supplementary Figs. 13b, c, and 14a, b), with an increase in GFP-2×FYVE and ATG18a-GFP targeting (Fig. 5c and Supplementary Table 5). These proteins form the autophagosome-like cup and ring structures that surround peroxisomes. These findings indicate that the light-induced peroxisome aggregates are specifically degraded via pexophagy. The peroxisomal aggregation in peup1/atg2 consists of oxidative peroxisomes with inactive catalase. Therefore, ATG18a recognises the oxidative peroxisomes through binding activity with PtdIns3P to degrade them.

ATG18a-GFP was occasionally localised to places other than peroxisomes (Fig. 2d), such as chloroplasts (Supplementary Fig. 18a–e) and undefined structures in the cell (Figs. 2a, d, 3a, d and Supplementary Fig. 6a and Supplementary Table 3), suggesting that some of the chloroplasts and other cellular materials are degraded by autophagy under light. In our tested-light conditions, ATG18a-GFP and GFP-2×FYVE recognised chloroplasts with lower chlorophyll fluorescence, presumably chloroplasts damaged under high-intensity light (Supplementary Fig. 18a–e). This is consistent with previous reports showing that high-intensity light induces ROS accumulation in chloroplasts, and subsequent degradation of damaged chloroplasts by autophagy (chlorophagy). Meanwhile, the relative intensity of H2-DCF from peroxisomes in peup1/atg2 and peup4/atg7 was about three times stronger than that from chloroplasts (Supplementary Fig. 17c, e). We also found that autophagy contributed slightly to the degradation of mitochondria, but to a lesser degree than pexophagy under light (Supplementary Fig. 15). We noticed that HSP70s were recovered in the pull-down assay of ATG18a-GFP (Supplementary Fig. 9 and Supplementary Table 2), implying the involvement of chaperone-mediated autophagy or microautophagy. Collectively, these findings suggest that various types of cellular components, mostly damaged peroxisomes, are degraded by autophagy under light.

**Plants have a unique mechanism for pexophagy**

Selective autophagy has been well studied in yeast and mammals, but less so in plants. The subcellular location of PtdIns3P synthesis during autophagy differs depending on the organisms and organelles to be degraded (e.g., vacuoles in yeast and omegasomes in mammals). In plants, the location of PtdIns3P synthesis, the origin of isolation membranes, and how ATGs participate in pexophagosome formation are unknown.
We showed that many dot structures of ATG18-GFP (Fig. 2a, e, f) and GFP-2×FYVE (Fig. 3a, e, f) localise to peroxisomes in *peup1/atg2* and *peup4/atg7*, suggesting that PtdIns3P is formed adjacent to the peroxisomes to attract ATG18a before the action of ATG2 and ATG7. The detailed analysis by electron microscope revealed that PtdIns3P and ATG18a were localised on both peroxisomes and phagophores adjacent to peroxisomes (Supplementary Fig. 11b). In wild type and *peup4/atg7*, we observed that a dot structure of ATG18-GFP or GFP-2×FYVE gradually change to a ring structure via a cup structure to engulf peroxisomes, but this change was not observed in *peup1/atg2* (Supplementary Figs. 7, 10 and Supplementary Videos 5, 6, 7, 9, 10). The aggregated peroxisomes were captured in invagination into vacuoles in *peup4/atg7* (Fig 5f, g, and Supplementary Fig. 16). Based on these results, we propose the following model for pexophagy (Fig. 6c): 1) peroxisomes with damaged catalase accumulate high levels of ROS, and PtdIns3P is generated on the peroxisome membrane or phagophores formed adjacent to peroxisome and ER, 2) ATG18a targets the PtdIns3P on the damaged peroxisomes, 3) pexophagosome is formed based on ATG18a and PtdIns3P with other autophagy factors, 4) pexophagosomes completely sequester damaged peroxisomes, and 5) pexophagosomes are incorporated into the vacuole.

We speculate that ROS generation is responsible for the induction of pexophagy, but it is still unclear how ROS generated in the peroxisome matrix are recognised for pexophagy. In human pexophagy, ataxia-telangiectasia mutated protein on the peroxisomal membrane senses ROS inside the peroxisome to induce pexophagy by mediating mTORC1 suppression and peroxin 5 (PEX5) phosphorylation. Plant pexophagy might also involve sensor protein(s) along with plant PEX proteins on the peroxisome membrane to induce pexophagy. In yeasts, receptors such as PpAtg30 and ScAtg36 interact with PEX3 and PEX14 to recognise peroxisomes to be degraded in pexophagy, but orthologues of these receptors are not found in plants. Alternatively, oxidised lipids on the peroxisome membrane may be the signal to induce pexophagosome formation, because they are the hallmark of oxidised peroxisomes. The accumulation mechanisms of PtdIns3P exist on both peroxisomes and phagophores. This is supported by the fact that multiple pathways for the accumulation of PtdIns3P are activated in autophagy. In mitophagy in mammalian cells, activation of phosphoinositide 3-kinase and inactivation of PTEN, a PtdIns3P phosphatase, occur on the membrane of initial phagophores, namely omegasomes, which are derived from the ER as platforms executing mitophagy. Recent studies have shown that phagophores in mammalian cells are generated from the contact site between the ER and mitochondria and in plant cells from the ER in which ATG5, ATG9, and ATG18 are localised. In yeast Saccharomyces cerevisiae, ATG2-ATG18 complex tethers PAS to ER for extending isolation membrane. We showed that the ER and phagophores were located adjacent to the high-density area in peroxisomes of *peup1/atg2* (Supplementary Fig. 11b, c) and *atg5* mutants. ATG18a and PI(3)P were localized to the area (Figs. 2, 3). These findings suggest that the initial phagophore generates at the site where the ER overlaps with a specific receptor and the PtdIns3P on peroxisomes in plant pexophagy, acting as a platform of PAS (Fig. 6c). ATG18 gathers on the PtdIns3P for extension of pexophagosomes with a lateral supply of isolation membrane from ER.
Leaf damage and peroxisome aggregation in \textit{atg9} are reduced compared with those in \textit{atg2}, \textit{atg5}, and \textit{atg7} under high-intensity (Supplementary Fig. 13) and normal light conditions \cite{11,14}, suggesting that the contribution of ATG9 in plant pexophagy is small, unlike in yeast and mammal pexophagy \cite{18,19,45,46}. ATG9 might not have a specific role in pexophagy, although it is generally required for autophagy in plants.

After initiation, the phagophore elongates to cover the peroxisome and become a pexophagosome, which then enters into vacuoles for degradation. Lack of autophagy causes accumulation of damaged peroxisomes and consequently indicates the aggregation of peroxisomes. ATG2 and ATG18a play an indispensable role in enveloping the degraded peroxisomes with ATG8-PE to form pexophagosomes (Fig. 6c). We provided the scheme of process in degradation and formation of the peroxisome aggregation in wild type, \textit{peup1/atg2}, and \textit{peup4/atg7} (Supplementary Fig 19). The difference phenotypes of peroxisome aggregates and dispersion (Fig. 1) may reflect a ATG function in formation of pexophagosome. ATG7 plays a role in the maturation of ATG8-PE as a ubiquitin-activating enzyme-like protein for generating autophagosomes \cite{57,58}. We previously observed ATG8a as dot structures close to degraded peroxisomes in \textit{peup1/atg2} and \textit{atg5}\cite{11,14}. Collectively, these data suggest ATG2, ATG5, ATG7, ATG18a, and ATG8-PE work cooperatively to generate complete pexophagosomes.

We found that the vacuolar membrane surrounded peroxisomes in \textit{peup4/atg7} (Fig. 5f–I and Supplementary Fig. 16a), suggesting possible microautophagy during the incorporation of pexophagosomes into the vacuole. Because the process of microautophagy seems incomplete in \textit{peup4/atg7}, ATG7 and ATG8-PE are probably required in microautophagy. Moreover, in \textit{peup4/atg7} cells, bulbs \cite{59}, spherical membrane structures of vacuoles, also interacted with peroxisomes at high frequency (Supplementary Fig. 16 and Supplementary Videos 11,12), suggesting their involvement in microautophagy. Taken together, these findings suggest that macro- and micro-pexophagy are induced under high-intensity light conditions.

We demonstrated that ATG18a-GFP selectively targets and surrounds peroxisomes to be degraded; this is the first observation of pexophagosomes forming from phagophores in plant cells. Hence, our analysis gives deep insight into the mechanism of autophagosome formation. Furthermore, our findings allow further understanding of how plants reduce ROS production via autophagy to improve photosynthetic efficiency and thus increase crop yield.

**Material And Methods**

**Plant material and growth condition**

Wild-type and transgenic plants were grown in a 16 h light/8 h dark cycle at 23 °C in an incubator (MLR-351, Sanyo Electric Co., Ltd., Japan). \textit{Arabidopsis thaliana} (L.) Heynh (Columbia, Col-0) and that expressing \textit{GFP-PTS1} (the GFP-PTS1 plant) or \textit{RFP-PTS1} (the RFP-PTS1 plant) \cite{60} were used as controls. The \textit{peup1}, \textit{peup2}, and \textit{peup4} (\textit{peups}) plants were previously screened as pexophagy mutants \cite{11}, and T-
DNA insertion lines atg2-1 (SALK_076727), atg5-1 (SAIL_129B07), atg7-2 (GABI_655B06), and atg9-3 (SALK_130796) were used for plant growth analysis. The peups expressing RFP-PTS1 (RP) were generated from F3 lines by crossing peups with the RFP-PTS1 plant. We produced the RFP-PTS1 plants expressing ATG18a-GFP or GFP-2×FYVE using the floral dip method with Agrobacterium tumefaciens (EHA101) harbouring the binary vector, pGW451-ATG18a or pGW452-2×FYVE. More than three independent lines that showed normal growth phenotype like Col-0 were selected (Supplementary Fig. 12c, d). The peups (RP) expressing ATG18a-GFP or GFP-2×FYVE were generated by crossing plants expressing both RFP-PTS1 and ATG18a-GFP or GFP-2×FYVE with peups (RP). These lines theoretically express the transgenes at the same level. Transgenic Arabidopsis expressing Venus-VAM3 (vacuolar membrane marker), or Mt-GFP (mitochondrial marker) were crossed with the RFP-PTS1 plant and peups (RP), respectively, to generate T3 homozygous lines.

**Plant growth analysis under high-intensity light conditions**

One week after germination on 0.8% (w/v) agar plates containing half-strength MS medium and 1% (w/v) sucrose at 23 °C in a 16 h light (50 µmol m⁻² s⁻¹)/8 h dark photoperiod, the plants were transferred to soil in 50 µmol m⁻² s⁻¹ white light (OSRAM FL25W White, Hitachi, Japan) at the same photoperiod for 2 weeks and then placed in incubators with white light of 50, 100, and 200 µmol m⁻² s⁻¹ for plant growth analysis (Supplementary Fig. 1). The plant growth analysis (Figs 4–6 and Supplementary Figs. 4, 13-15, 17,18) involved irradiation with blue (450 nm) and red (640 nm) light using a LED equipment (ISC-150×150-H4RB45; CCS, Japan) with a power supply (ISC-201-2; CCS, Japan) in normal and high-intensity light conditions at 100 and 1000 µmol m⁻² s⁻¹, respectively. After the plants were grown at 23 °C in the 16 h light (100 µmol m⁻² s⁻¹)/8 h dark cycle for 3 weeks on 0.8 % (w/v) agar containing 1% (w/v) sucrose and 1 ´ MS salt, the plant samples were used for biochemical analysis.

**Vector construction**

Binary vectors pGW451-ATG18a-G3-GFP and pGW452-G3-GFP-2×FYVE were constructed using the Gateway system (Thermo Fisher Scientific, Waltham, MA, USA) to transform the RFP-PTS1 plant. Adapter-tagged cDNA of AtATG18a (At3g62770, accession no.: NM_116142) was generated by PCR, amplifying the corresponding region using the following primer set: F: 5'-TACAAAAAAGCAGGCTTCATGGCCACCGTATCTTCTTC-3', R: 5'-GTACAAGAAAGCTGGGTTGAAAACTGAAGGCGGTTTCAGA-3' for ATG18a, which was then recombined with the pDONR™221 vector.

Adapter-tagged cDNA of the FYVE domain was generated by PCR, amplifying the corresponding region using the following primer set: attB1-adapter, 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTC-3'; attB2-adapter, 5'-GGGGACCACTTTGATCAAGAAGCTGGTT-3' using the pBluescript KS (-) (Stratagene) vector containing the nucleotide sequence of 2×FYVE domain with attB1 and attB2 as templates and then recombining with the pDONR™221 vector. The nucleotide sequences of two FYVE domains, attB1-FYVE and FYVE-attB2, were separately inserted in the same pBluescript KS (-) vector.
two steps using the FYVE region of *Mus musculus* HGF-regulated tyrosine kinase substrate (Hgs; accession no.: NM_001159328) as the cDNA template. Two primers sets, F1: 5'-AAGTCGACTCATAAAGCAGGCYYCGAAGTGATGCCATGTTCGCTG-3' and R1: 5'-AAAAGCTTGACCCTTTGAGCTCTTGTTCAGCTGCTCATA-3' for *attB1*-FYVE; and F2: 5'-AAAAAGCTTCTGAAAGTGATGCCATGTTCGCTGCTGAAA-3' and R2: 5'-AAGATTCGTACAAGAAGCTGGGTGCTCCTCTTGTTCAGCTGCTCATA-3' for FYVE-*attB2* were used to amplify the corresponding region.

**Imaging analysis**

A confocal laser scanning microscope (LSM 510, Zeiss, Germany) with a 40× or 63x objective was used for imaging analyses of peroxisomes and for determining the intracellular distribution of fluorescent proteins as described previously. The excitation and emission wavelengths for the images were 488 nm and 492-570 nm, respectively, for GFP, and 516 nm and 600-625 nm, respectively, for RFP. Time-lapse images were obtained for 250-300 s with a temporal resolution of 5 s, and movie files were generated with Fiji (ImageJ, NIH public domain). The number of cells and organelles were counted using the Analyze Particles and Cell Counter plugins equipped in Fiji. The size of peroxisome aggregation was measured manually using the polygon selection tool in Fiji after the images were magnified three-fold for precise selection of the periphery. The pexophagosome around peroxisomes in *peup4* targeted by ATG18a-GFP (Fig. 2h) was identified by conducting mathematical morphology analysis based on the time-lapse images. Fluorescence intensity (Figs. 2g, 3g, 5e, 6a and Supplementary Fig. 18b) was measured using the Plot Profile plugin equipped in Fiji. FRAP analysis (Supplementary Fig. 8) was performed using LSM510 with an Ar laser (488 nm) at 50 % intensity to induce photobleaching. Images were obtained every 1 s, and then fluorescence intensity was measured using Fiji.

**Measurement of chlorophyll content and photosynthetic efficiency**

Chlorophyll content (Supplementary Fig. 1b) was measured as previously described using the rosette leaves adapted to each light intensity. Photosynthetic efficiency (Supplementary Fig. 1c) was measured as the maximum yield of photosynthesis system II using a photosynthesis yield analyser (MINI-PAM; Walz, Effeltrich, Germany) using at least three leaves from five plants after they were adapted to each light intensity. Three independent experiments were performed.

**Electron microscopy analysis**

Electron microscopy analysis was performed as described previously. Three-week-old wild-type and *peup4* plants were analysed for catalase accumulation (Supplementary Figs. 2 and 11c), chloroplast and peroxisome membranes (Supplementary Fig. 4e), and mitochondria (Supplementary Fig. 15b). Plant leaves were fixated in 4% (w/v) paraformaldehyde, 1% (w/v) glutaraldehyde, and 0.06 M sucrose in 0.05 M cacodylate buffer (pH 7.4). Immunoelectron microscopy analyses with antibodies against GFP and
peroxisomal proteins [malate synthase (MS), isocitrate lyase (ICL), glycolate oxidase (GO), hydroxypyruvate reductase (HPR), and catalase (CAT)] were performed as described previously 11,70.

NBT and H2-DCF staining

Nitro blue tetrazolium (NBT) and 2,7'-dichlorodihydrofluorescein (H2-DCF) staining were performed as follows: rosette leaves of GFP-PTS1, peup1, and peup4 were immediately submerged in NBT (Sigma-Aldrich) solution for 1 h, and then chlorophyll was repeatedly removed with 100 % ethanol in 95 °C water for 10 min and washed with pure water. In the case of H2-DCF staining, the leaves were submerged in 10 µM H2-DCF-DA (Thermo Fisher Scientific) for 10 min and then washed once with pure water 33,34. At least three independent experiments were performed.

Immunoblot analysis

Immunoblotting was performed as described previously 11. Total proteins of wild-type, peups, atgs, and various transgenic plants grown under different light intensities for 1–2 d were extracted with extraction buffer containing 10 mM HEPES-KOH (pH 8.0) and a protease inhibitor cocktail (Roche). Then, total proteins were fractionated into supernatant and pellet by centrifugation at 20,000 ´ g for 10 min at 4 °C. The pellets were washed with extraction buffer twice, followed by solubilisation with extraction buffer containing 1% (w/v) SDS. Each 10 µg of total protein was separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore) in a semidy electroblotting system (BioCraft). Then, immunoblot analyses were performed using antibodies against peroxisomal proteins catalase (CAT), peroxin 14 (PEX14), GO, ascorbate peroxidase (APX), and HPR 11, as well as against mitochondrial proteins cytochrome c oxidase 2 (COXII) (Agrisera, Sweden) and serine hydroxymethyltransferase (SHMT) (Agrisera, Sweden). Signal intensities of bands in the immunoblot image were quantified using Dot Blot Analysis in Fiji.

Mass spectrum analysis

Total protein was extracted from peup1 expressing ATG18a-GFP or GFP grown in light with 1 mL of lysis buffer [50 mM HEPES-KOH (pH 7.5), 0.15 M NaCl, 0.5% (v/v) Triton X-100, and 0.1% (v/v) Tween 20]. ATG18a-GFP-binding proteins were obtained by immunoprecipitation using µMACS Anti-GFP MicroBeads and µMACS columns (Miltenyi Biotec K.K., USA) 71. The eluted fraction was assessed by immunoblot analysis using anti-GFP antibody to detect GFP or ATG18a-GFP (Supplementary Fig. 9a). ATG18a-GFP/GFP-binding proteins were subjected to SDS-PAGE following in-gel digestion. Collected peptides were analysed using nano-LC-MS/MS (LTQ Orbitrap XL; Thermo Fisher Scientific) 72. The obtained spectra were searched against the TAIR 10 Arabidopsis protein database (version 20101214) with Mascot server (version 2.3.02, Matrix Science, London, UK) 73. The list of identified proteins is shown in Supplementary Table 2. The experiments were repeated three times.

Lipid binding assay
The binding ability of ATG18a-GFP to PtdIns3P was determined using PIP Strips P-6001 (Echelon Biosciences Inc., USA) according to the manufacturer’s instruction and Tamura et al. (2013) 31. ATG18a-GFP was isolated as a crude extract from peup1 expressing ATG18a-GFP and incubated with PIP strips for 3 h at 23 °C after removing debris by centrifugation at 1,000 ´ g for 5 min. After two washes with TBS containing 0.1% (v/v) Tween 20, the binding of ATG18a-GFP to lipids was detected using an antibody against GFP and ImageQuant LAS4000 (GE Healthcare) at high sensitivity mode.

Declarations

Acknowledgements

We thank Dr. Shunichi Takahashi (National Institute for Basic Biology) and Dr. Murray Badger (Australian National University) for helpful discussion about photoinhibition and photosynthetic efficiency in pexophagy mutants. We also thank Dr. Tsuyoshi Nakagawa (University of Shimane) for providing Gateway vectors pGWB551 and pGWB552, Dr. Shinichi Arimura (University of Tokyo) and Dr. Tomohiro Uemura (Ochanomizu University) for kindly providing the transgenic line expressing Mt-GFP and the plasmid hovering VAM3 gene, and Bioimaging Facility in National Institute for Basic Biology (NIBB) and NIBB Bio Resource Center for technical support. This work was supported by a Grant-in-Aid for Scientific Research on Innovative Areas to M.N. (no. 22120007) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT); by Grants-in-Aid for Scientific Research to Y.H. and K.O. (no. 17K07467), to M.N. (no. 20370024), to S.M. (nos. 26440157 and 20570045), and to I.H.-N. (nos. 15H05776 and 22000014) from Japan Society for the Promotion of Science (JSPS); by the Japan Science and Technology Agency Exploratory Research for Advanced Technology program (JST-ERATO) to K.N. (no. JPMJER1602); by a SONATA-BIS Grant to S.G.-Y. (UMO-2019/34/E/NZ3/00299) from National Science Centre Poland; by a TEAM Grant to K.Ya. (TEAM/2017-4/41) from the Foundation for Polish Science; by the Wyeth Foundation to M.N. and I.H.-N.; and by the Hirao Taro Foundation of KONAN GAKUEN for Academic Research to I.H.-N.

Author contributions

K.O., M.S., K.Yo., Y.H., S.G.-Y., S.M., K.Ya., Y.O, K. N, and M.N. designed the study. K.O., S.G.-Y., M.S., A.T., K.S., H.U., and S.M. performed research. Y.H., M.K., and K.S. performed EM analysis. Y.K. performed mathematical morphology analysis. D.T., and M.U. performed protein mass spectrometry analysis. K.O., S.G.-Y., K.Yo., A.K., H.U., I.H.-N., and S.M. generated transgenic plants and performed plant growth analysis. All the authors analysed the data and wrote the manuscript.

References

1. Oikawa K, Hayashi M, Hayashi Y, Nishimura M. Re-evaluation of physical interaction between plant peroxisomes and other organelles using live-cell imaging techniques. J Integr Plant Biol 61, 836–852 (2019).
2. Kozaki K, Takeba, G. Photorespiration protects C3 plants from photooxidation. *Nature* **384**, 557–560 (1996).

3. Takahashi S, Bauwe H, Badger M. Impairment of the photorespiratory pathway accelerates photoinhibition of photosystem II by suppression of repair but not acceleration of damage processes in Arabidopsis. *Plant Physiol* **144**, 487–494 (2007).

4. Willekens H, *et al.* Catalase is a sink for H$_2$O$_2$ and is indispensable for stress defence in C3 plants. *EMBO J* **16**, 4806–4816 (1997).

5. Corpas FJ, Barroso JB, del Rio LA. Peroxisomes as a source of reactive oxygen species and nitric oxide signal molecules in plant cells. *Trends Plant Sci* **6**, 145–150 (2001).

6. Sandilio LM, Romero-Puertas MC. Peroxisomes sense and respond to environmental cues by regulating ROS and RNS signalling networks. *Ann Bot* **116**, 475–485 (2015).

7. Foyer CH, Bloom AJ, Queval G, Noctor G. Photorespiratory metabolism: genes, mutants, energetics, and redox signaling. *Annu Rev Plant Biol* **60**, 455–484 (2009).

8. Apel K, Hirt H. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu Rev Plant Biol* **55**, 373–399 (2004).

9. Noctor G, Foyer CH. Intracellular redox compartmentation and ROS-related communication in regulation and signaling. *Plant Physiol* **171**, 1581–1592 (2016).

10. Del Rio LA, Lopez-Huertas E. ROS generation in peroxisomes and its role in cell signaling. *Plant Cell Physiol* **57**, 1364–1376 (2016).

11. Shibata M, *et al.* Highly oxidized peroxisomes are selectively degraded via autophagy in Arabidopsis. *Plant Cell* **25**, 4967–4983 (2013).

12. Zhang J, *et al.* A tuberous sclerosis complex signalling node at the peroxisome regulates mTORC1 and autophagy in response to ROS. *Nat Cell Biol* **15**, 1186–1196 (2013).

13. Pérez-Pérez ME, Lemaire SD, Crespo JL. Reactive oxygen species and autophagy in plants and algae. *Plant Physiol* **160**, 156–164 (2012).

14. Yoshimoto K, *et al.* Organ-specific quality control of plant peroxisomes is mediated by autophagy. *J Cell Sci* **127**, 1161–1168 (2014).

15. Xie Z, Klionsky DJ. Autophagosome formation: core machinery and adaptations. *Nat Cell Biol* **9**, 1102–1109 (2007).

16. Mizushima N, Yoshimori T, Ohsumi Y. The role of Atg proteins in autophagosome formation. *Annu Rev Cell Dev Biol* **27**, 107–132 (2011).

17. Liu Y, Bassham DC. Autophagy: pathways for self-eating in plant cells. *Annu Rev Plant Biol* **63**, 215–237 (2012).

18. Ohsumi Y. Historical landmarks of autophagy research. *Cell Res* **24**, 9–23 (2014).

19. Farre JC, Subramani S. Mechanistic insights into selective autophagy pathways: lessons from yeast. *Nat Rev Mol Cell Biol* **17**, 537–552 (2016).
20. Nair U, Cao Y, Xie Z, Klionsky DJ. Roles of the lipid-binding motifs of Atg18 and Atg21 in the cytoplasm to vacuole targeting pathway and autophagy. *J Biol Chem* **285**, 11476–11488 (2010).

21. Obara K, Ohsumi Y. PtdIns 3-kinase orchestrates autophagosome formation in yeast. *J Lipids* 2011, 498768 (2011).

22. Oku M, Sakai Y. Pexophagy in yeasts. *Biochim Biophys Acta* **1863**, 992–998 (2016).

23. Anding AL, Baehrecke EH. Cleaning house: selective autophagy of organelles. *Dev Cell* **41**, 10–22 (2017).

24. Filomeni G, De Zio D, Cecconi F. Oxidative stress and autophagy: the clash between damage and metabolic needs. *Cell Death Differ* **22**, 377–388 (2015).

25. Sinclair AM, Trobacher CP, Mathur N, Greenwood JS, Mathur J. Peroxule extension over ER-defined paths constitutes a rapid subcellular response to hydroxyl stress. *Plant J* **59**, 231–242 (2009).

26. Brunkard JO, Runkel AM, Zambryski PC. Chloroplasts extend stromules independently and in response to internal redox signals. *Proc Natl Acad Sci USA* **112**, 10044–10049 (2015).

27. Xiong Y, Contento AL, Nguyen PQ, Bassham DC. Degradation of oxidized proteins by autophagy during oxidative stress in Arabidopsis. *Plant Physiol* **143**, 291–299 (2007).

28. Kimori Y, Hikino K, Nishimura M, Mano S. Quantifying morphological features of actin cytoskeletal filaments in plant cells based on mathematical morphology. *J Theor Biol* **389**, 123–131 (2016).

29. Xiong Y, Contento AL, Bassham DC. AtATG18a is required for the formation of autophagosomes during nutrient stress and senescence in *Arabidopsis thaliana*. *Plant J* **42**, 535–546 (2005).

30. Krick R, Tolstrup J, Appelles A, Henke S, Thumm M. The relevance of the phosphatidylinositolphosphat-binding motif FRRGT of Atg18 and Atg21 for the Cvt pathway and autophagy. *FEBS Lett* **580**, 4632–4638 (2006).

31. Tamura N, Oku M, Ito M, Noda NN, Inagaki F, Sakai Y. Atg18 phosphoregulation controls organellar dynamics by modulating its phosphoinositide-binding activity. *J Cell Biol* **202**, 685–698 (2013).

32. Vermeer JE, et al. Visualization of PtdIns3P dynamics in living plant cells. *Plant J* **47**, 687–700 (2006).

33. de Torres Zabala M, et al. Chloroplasts play a central role in plant defence and are targeted by pathogen effectors. *Nat Plants* **1**, 15074 (2015).

34. Yamauchi S, et al. Autophagy controls reactive oxygen species homeostasis in guard cells that is essential for stomatal opening. *Proc Natl Acad Sci USA* **116**, 19187–19192 (2019).

35. Foyer CH NG. Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. *Physiologia Plantarum* **119**, 355–364 (2003).

36. Oikawa K, et al. Physical interaction between peroxisomes and chloroplasts elucidated by in situ laser analysis. *Nat Plants* **1**, 15035 (2015).

37. Polson HE, et al. Mammalian Atg18 (WIPI2) localizes to omegasome-anchored phagophores and positively regulates LC3 lipidation. *Autophagy* **6**, 506–522 (2010).
38. Proikas-Cezanne T, Takacs Z, Donnes P, Kohlbacher O. WIPI proteins: essential PtdIns3P effectors at the nascent autophagosome. *J Cell Sci* **128**, 207–217 (2015).
39. Izumi M, Ishida H, Nakamura S, Hidema J. Entire photodamaged chloroplasts are transported to the central vacuole by autophagy. *Plant Cell* **29**, 377–394 (2017).
40. Tekirdag K, Cuervo AM. Chaperone-mediated autophagy and endosomal microautophagy: Joint by a chaperone. *J Biol Chem* **293**, 5414–5424 (2018).
41. Mizushima N, Komatsu M. Autophagy: renovation of cells and tissues. *Cell* **147**, 728–741 (2011).
42. Cheng J, *et al.* Yeast and mammalian autophagosomes exhibit distinct phosphatidylinositol 3-phosphate asymmetries. *Nat Commun* **5**, 3207 (2014).
43. Roberts R, Ktistakis NT. Omegasomes: PI3P platforms that manufacture autophagosomes. *Essays Biochem* **55**, 17–27 (2013).
44. Nascimbeni AC, Codogno P, Morel E. Phosphatidylinositol-3-phosphate in the regulation of autophagy membrane dynamics. *FEBS J* **284**, 1267–1278 (2017).
45. Stolz A, Ernst A, Dikic I. Cargo recognition and trafficking in selective autophagy. *Nat Cell Biol* **16**, 495–501 (2014).
46. Carlsson SR, Simonsen A. Membrane dynamics in autophagosome biogenesis. *J Cell Sci* **128**, 193–205 (2015).
47. Young PG, Bartel B. Pexophagy and peroxisomal protein turnover in plants. *Biochim Biophys Acta* **1863**, 999–1005 (2016).
48. Subramani S. A mammalian pexophagy target. *Nat Cell Biol* **17**, 1371–1373 (2015).
49. Motley AM, Nuttall JM, Hettema EH. Pex3-anchored Atg36 tags peroxisomes for degradation in *Saccharomyces cerevisiae*. *EMBO J* **31**, 2852–2868 (2012).
50. Maehama T, Taylor GS, Dixon JE. PTEN and myotubularin: novel phosphoinositide phosphatases. *Annu Rev Biochem* **70**, 247–279 (2001).
51. Nguyen TN, Padman BS, Lazarou M. Deciphering the molecular signals of PINK1/Parkin mitophagy. *Trends Cell Biol* **26**, 733–744 (2016).
52. Harper JW, Ordureau A, Heo JM. Building and decoding ubiquitin chains for mitophagy. *Nat Rev Mol Cell Biol* **19**, 93–108 (2018).
53. Hamasaki M, *et al.* Autophagosomes form at ER-mitochondria contact sites. *Nature* **495**, 389–393 (2013).
54. Le Bars R, Marion J, Le Borgne R, Satiat-Jeunemaitre B, Bianchi MW. ATG5 defines a phagophore domain connected to the endoplasmic reticulum during autophagosome formation in plants. *Nat Commun* **5**, 4121 (2014).
55. Zhuang X, *et al.* ATG9 regulates autophagosome progression from the endoplasmic reticulum in *Arabidopsis*. *Proc Natl Acad Sci USA* **114**, E426–E435 (2017).
56. Kotani, T., Kirisako, H., Koizumi, M., Ohsumi, Y. & Nakatogawa, H. The Atg2-Atg18 complex tethers pre-autophagosomal membranes to the endoplasmic reticulum for autophagosome formation. *Proc
57. Mizushima N, et al. A protein conjugation system essential for autophagy. *Nature* 395, 395–398 (1998).

58. Komatsu M, et al. Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. *J Cell Biol* 169, 425–434 (2005).

59. Saito C, et al. A complex and mobile structure forms a distinct subregion within the continuous vacuolar membrane in young cotyledons of Arabidopsis. *Plant J* 29, 245–255 (2002).

60. Mano S, Nakamori C, Hayashi M, Kato A, Kondo M, Nishimura M. Distribution and characterization of peroxisomes in Arabidopsis by visualization with GFP: dynamic morphology and actin-dependent movement. *Plant Cell Physiol* 43, 331–341 (2002).

61. Zhang X, Henriques R, Lin SS, Niu QW, Chua NH. Agrobacterium-mediated transformation of Arabidopsis thaliana using the floral dip method. *Nat Protoc* 1, 641–646 (2006).

62. Ebine K, et al. A SNARE complex unique to seed plants is required for protein storage vacuole biogenesis and seed development of Arabidopsis thaliana. *Plant Cell* 20, 3006–3021 (2008).

63. Goto-Yamada S, et al. Sucrose starvation induces microautophagy in plant root cells. *Front Plant Sci* 10, 1604 (2019).

64. Arimura S, Yamamoto J, Aida GP, Nakazono M, Tsutsumi N. Frequent fusion and fission of plant mitochondria with unequal nucleoid distribution. *Proc Natl Acad Sci USA* 101, 7805–7808 (2004).

65. Nakagawa T, et al. Improved gateway binary vectors: high-performance vectors for creation of fusion constructs in transgenic analysis of plants. *Biosci Biotechnol Biochem* 71, 2095–2100 (2007).

66. Sankaran VG, Klein DE, Sachdeva MM, Lemmon MA. High-affinity binding of a FYVE domain to phosphatidylinositol 3-phosphate requires intact phospholipid but not FYVE domain oligomerization. *Biochemistry* 40, 8581–8587 (2001).

67. Schindelin J, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods* 9, 676–682 (2012).

68. Asakura Y, et al. Maize mutants lacking chloroplast FtsY exhibit pleiotropic defects in the biogenesis of thylakoid membranes. *Plant Cell* 16, 201–214 (2004).

69. Yamaguchi K, Nishimura M. Reduction to below threshold levels of glycolate oxidase activities in transgenic tobacco enhances photoinhibition during irradiation. *Plant Cell Physiol* 41, 1397–1406 (2000).

70. Hayashi Y, Hayashi M, Hayashi H, Hara-Nishimura I, Nishimura M. Direct interaction between glyoxysomes and lipid bodies in cotyledons of the Arabidopsis thaliana ped1 mutant. *Protoplasma* 218, 83–94 (2001).

71. Tamura K, Fukao Y, Iwamoto M, Haraguchi T, Hara-Nishimura I. Identification and characterization of nuclear pore complex components in *Arabidopsis thaliana*. *Plant Cell* 22, 4084–4097 (2010).

72. Takahashi D, Li B, Nakayama T, Kawamura Y, Uemura M. Shotgun proteomics of plant plasma membrane and microdomain proteins using nano-LC-MS/MS. *Methods Mol Biol* 1072, 481–498
73. Takahashi D, Kawamura Y, Uemura M. Cold acclimation is accompanied by complex responses of glycosylphosphatidylinositol (GPI)-anchored proteins in Arabidopsis. *J Exp Bot* 67, 5203–5215 (2016).

74. Scherz-Shouval R, Shvets E, Fass E, Shorer H, Gil L, Elazar Z. Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. *EMBO J* 26, 1749–1760 (2007).