SHORT REPORT

Organ-specific quality control of plant peroxisomes is mediated by autophagy

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ABSTRACT

Peroxisomes are essential organelles that are characterized by the possession of enzymes that produce hydrogen peroxide (H₂O₂) as part of their normal catalytic cycle. During the metabolic process, peroxisomal proteins are inevitably damaged by H₂O₂ and the integrity of the peroxisomes is impaired. Here, we show that autophagy, an intracellular process for vacuolar degradation, selectively degrades dysfunctional peroxisomes. Marked accumulation of peroxisomes was observed in the leaves but not roots of autophagy-related (ATG)-knockout Arabidopsis thaliana mutants. The peroxisomes in leaf cells contained markedly increased levels of catalase in an insoluble and inactive aggregate form. The chemically inducible complementation system in ATG5-knockout Arabidopsis provided the evidence that these accumulated peroxisomes were delivered to vacuoles for degradation by autophagy. Interestingly, autophagosomal membrane structures specifically recognized the abnormal peroxisomes at the site of the aggregates. Thus, autophagy is essential for the quality control of peroxisomes in leaves and for proper plant development under natural growth conditions.

KEY WORDS: Autophagy, Organelle degradation, Plant leaf, Quality control

INTRODUCTION

Peroxisomes are essential organelles characterized by the possession of enzymes that produce hydrogen peroxide (H₂O₂) as part of their normal catalytic cycle (De Duve and Baudhuin, 1966). In plant leaves, two major types of peroxisomes play crucial roles in the postgerminative development of seedlings and photosynthesis (Beevers, 1979). A type of peroxisome, the glyoxysome, is involved in fatty acid β-oxidation to convert storage oil into carbohydrates in cotyledons, the embryonic first leaves, to support the postgerminative growth of seedlings until the commencement of photoautotrophic growth. The other prominent type of plant peroxisome, the leaf peroxisome, is unique in that it plays an important role in photorespiration (i.e. the photosynthesis-related oxidative carbon cycle). In both metabolic pathways, especially in photorespiration, H₂O₂ is generated by oxidative reactions (Foyer and Noctor, 2003) and is scavenged by catalase, which is present in both types of peroxisomes (del Rio et al., 2006). Proteins in peroxisomes are damaged by reactive oxygen species (ROS) produced during such peroxisomal oxidation processes. Plants with impaired peroxisome function exhibit a defect in seedling development and have decelerated growth phenotypes (Reumann and Weber, 2006; Fulda et al., 2004, Rylott et al., 2006), indicating that the maintenance and turnover of peroxisomes are important for plant development and growth. Despite their importance, the mechanism by which the basal quality of peroxisomes is maintained is unknown.

Macroautophagy (referred to hereafter as autophagy) is an evolutionarily conserved intracellular process in eukaryotes, which involves the degradation of cytoplasmic constituents and organelles by sequestration in double-membraned vesicles, the autophagosomes (Mizushima et al., 2011). In yeast, phytopathogens and animal cells, there is a type of autophagy that is specific for peroxisome degradation, known as ‘pexophagy’. This involves the removal of excess or redundant peroxisomes under particular artificial growth conditions in which peroxisomal functions are no longer required (Sakai et al., 2006; Asakura et al., 2009; Iwata et al., 2006). However, the role of pexophagy in the quality control of peroxisomes remains obscure.

RESULTS AND DISCUSSION

We investigated the behavior of peroxisomes in ATG-knockout Arabidopsis thaliana (atg mutants) expressing green fluorescent protein (GFP) fused with PTS1 (Mano et al., 2002), a peroxisomal targeting signal. The number of peroxisomes was increased in the leaves of all atg mutants examined (Fig. 1A, upper-right panel; Fig. 1B; supplementary material Fig. S1A,B). By contrast, there was no obvious difference in the number of peroxisomes in roots between wild-type plants and atg mutants (Fig. 1A, lower panels; supplementary material Fig. S1C,D). Western blotting analysis using antibodies against the representative peroxisomal protein catalase further confirmed these imaging data. Although there were no substantial differences in the transcript levels of catalase genes (supplementary material Fig. S2A; Frugoli et al., 1996), the catalase protein levels were markedly increased in atg5 leaves, but not in the roots, compared to wild-type controls (Fig. 1C). These results suggest that the number of photosynthesis-related peroxisomes is increased in atg mutants.
As it has been reported that overexpression of PEX11 genes, which encode peroxisomal membrane proteins, causes an increase in peroxisome number (Orth et al., 2007), we checked the transcript levels of the PEX11 genes in atg mutants. However, the transcription levels of PEX11 genes in atg mutants were similar to those in wild type (supplementary material Fig. S2B). We further confirmed that peroxisome numbers were increased even in NahG atg5 plant leaves (supplementary material Fig. S3), in which endogenous salicylic acid (SA) is depleted and consequently the early senescence phenotype is suppressed (Yoshimoto et al., 2009). This indicated that the increase in peroxisome number was not caused by side effects, such as highly accumulated SA or the onset of senescence in atg mutants. These results further support the view that peroxisomes in leaves undergo less degradation in atg mutants, resulting in an increased number of peroxisomes.

We next examined whether autophagy preferentially degrades only peroxisomes and not other organelles. For this, western blotting analysis was performed using antibodies against representative organelle marker proteins to compare the protein levels of each organelle between wild-type and atg5 leaves. As shown in Fig. 1D,E, only peroxisomal proteins were increased in atg5 leaves. In contrast to the increase in peroxisome number, there was no increase in the number of mitochondria in atg5 leaves (supplementary material Fig. S4), strongly suggesting that leaf peroxisomes are selectively degraded by autophagy.

Next, the fine structure of peroxisomes in the leaves of atg mutants was examined by electron microscopy (EM) (Fig. 2A). Consistent with the results of fluorescence imaging (Fig. 1A, upper-right panel), many peroxisomes were present in atg mutants. In addition, we found that many peroxisomes in atg mutants contained some electron-dense regions (Fig. 2A, right panel; Fig. 2B; supplementary material Table S1).

As the levels of catalase protein were markedly increased in atg5 leaves compared to those of other peroxisomal proteins (Fig. 1E), we examined the intracellular states of the highly accumulated catalase by immuno-EM analysis (Fig. 2C). Gold particles conjugated with anti-catalase antibodies were evenly distributed in normal peroxisomes. In abnormal peroxisomes, however, the density of gold particles was markedly elevated in...
the electron-dense regions and, in such peroxisomes, the density of gold particles was reduced in normal regions, indicating that catalase was concentrated in the electron-dense regions. The electron-dense regions of atg mutant peroxisomes had the appearance of structures that have been reported as aggregates of dysfunctional peroxisome-matrix proteins in the peroxisomal Lon protease-deficient fungus (Bartoszewska et al., 2012). Based on these observations, we hypothesized that the electron-dense regions are dysfunctional protein aggregates, one of the major components of which is catalase. Indeed, there were differences of dysfunctional peroxisome-matrix proteins in the peroxisomal Lon protease-deficient fungus (Bartoszewska et al., 2012). Based on these observations, we hypothesized that the electron-dense regions are dysfunctional protein aggregates, one of the major components of which is catalase. Indeed, there were differences

Fig. 2. See next page for legend.
in the biochemical features of catalase between wild-type and atg5 leaves; high levels of insoluble catalase were present in atg5 leaves (Fig. 2D), and its enzyme activity was substantially lower than that of soluble catalase (Fig. 2E), indicating that atg mutants accumulated an inactive form(s) of catalase. Furthermore, the redox status of a proportion of peroxisomes in the atg5 mutant was disturbed, showing a more oxidized state than wild-type ones (Fig. 2F). These results suggest that peroxisomes with electron-dense regions in atg mutants are dysfunctional. We also found that peroxisomes in atg mutant leaves did not show the appropriate intracellular localization (Fig. 2G). Leaf peroxisomes are usually tightly attached to chloroplasts and/or mitochondria, where they cycle metabolites among these organelles during photorespiration. In wild-type leaves, almost 80% of peroxisomes were located within a distance of 0.1 μm from chloroplasts and/or mitochondria. In atg mutant leaves, however, more than 45% of peroxisomes were separated from these organelles and were often clustered together, which would also lead to a reduction of peroxisomal capability (Prestele et al., 2010). Leaves of atg mutants were indeed hypersensitive to the catalase inhibitor 3-aminotriazole (Fig. 2H), even though the levels of catalase protein were increased, and this result closely correlated with the accumulation of inactive form(s) of catalase and the mislocalization of peroxisomes in atg mutants.

Consistent with our findings, atg mutants showed characteristic phenotypes similar to those observed in peroxisomal-function-defective plants. Arabidopsis photorespiratory mutants show stunted or decelerated growth under standard atmospheric conditions but grow normally in air enriched in CO₂, when photorespiration is suppressed (Reumann and Weber, 2006). Likewise, the decelerated growth phenotype observed in the atg5 mutant was partially suppressed under high CO₂ conditions (Fig. 2I). We also examined postgerminative seedling establishment on sugar-free medium as an index of peroxisomal ability. As Arabidopsis cotyledons catabolize fatty acids into sugars in glyoxysomes, a decline in peroxisomal activity would result in a lower percentage of seedlings developing true leaves on sugar-free medium (Fulda et al., 2004; Rylott et al., 2006). Indeed, the atg5 mutant showed a lower frequency of seedling development without supplemental sugars. Cotyledons of ~50% of atg5 seedlings did not fully expand and the production of true leaves was prevented, whereas this was not the case in wild-type seedlings (Fig. 2J). This growth defect in atg5 was rescued by exogenous application of sugars (Fig. 2K).

The accumulation of abnormal peroxisomes in atg mutants prompted us to examine whether autophagy transports peroxisomes to the vacuolar lumen for degradation. For this purpose, an atg5 transgenic was generated. In this mutant, the autophagic defect can be complemented through induction of ATG5 gene expression by treatment with 17-β-estradiol, and peroxisomes can be visualized by monitoring GFP fluorescence by confocal fluorescence microscopy. The number of peroxisomes was reduced in the transgenic plants after 17-β-estradiol treatment (Fig. 3A, upper-right panel), whereas control leaves still retained a large number of peroxisomes (Fig. 3A, upper-left panel). To confirm that peroxisomes were delivered into the vacuolar lumen by autophagy, transgenic leaves were incubated in the dark after 17-β-estradiol treatment as GFP fluorescence cannot be detected in the vacuolar lumen in plants that are kept in the light (Tamura et al., 2003). The number of peroxisomes was reduced in 17-β-estradiol-treated leaves, and the vacuolar lumens in these cells contained GFP (Fig. 3A, lower right panel). When plant cells are treated with the V-ATPase inhibitor concanamycin A, autophagic bodies can also be detected inside the vacuolar lumen as small randomly moving vesicles (Yoshimoto et al., 2004). In concanamycin-A-treated cells without 17-β-estradiol treatment, in which autophagy is defective, peroxisomes were detected in the cytoplasm and did not show much movement, sometimes showing rectilinear motion along the cytoskeleton (supplementary material Movie 1). After 17-β-estradiol treatment, however, highly mobile peroxisomes were observed (supplementary material Movie 2), suggesting that peroxisomes were present in autophagic bodies within the vacuole. These results indicate that peroxisomes are delivered to the vacuole by autophagy for degradation.

We then sought to determine the mechanism by which selectivity in the degradation of plant peroxisomes by autophagy is guaranteed. On EM images, some of the peroxisomes in atg mutants appeared to be fragmented and consisted only of electron-dense material (Fig. 2A,C). This might explain the marked accumulation of catalases in atg5 leaves (Fig. 1E). A proportion of peroxisomes containing protein aggregates appeared to be segregated or ‘torn off’ from the whole peroxisome by unknown mechanisms (Fig. 3B), thus dysfunctional peroxisomal proteins could be selectively degraded by autophagy. In addition, the data for the frequency of peroxisomes with electron-dense regions indicated that the increased number of peroxisomes in atg mutants was mainly due to the increase of peroxisomes with electron-dense regions...
(supplementary material Table S1), suggesting that there is selective degradation of abnormal peroxisomes in wild-type leaves. This selectivity was further confirmed by the results of the EM analyses in this study. Examination of the fine structures of peroxisomes in atg mutants revealed an electron-lucent membrane structure, which was located adjacent to the endoplasmic reticulum (ER), occasionally present beside peroxisomes in atg2 leaves (Fig. 3C, black arrow). Surprisingly, these membrane structures were always tightly attached to the sites of the electron-dense regions (supplementary material Table S3). In a yeast atg2 mutant, many Atg proteins, including Atg8, accumulated at high levels in pre-autophagosomal structures (Suzuki et al., 2007), suggesting that an early autophagic structure called the ‘isolation membrane’ forms in the atg2 mutant but this mutant cannot complete autophagosome formation. In addition, it has been reported that the ER is one of the sources of isolation membranes in mammalian cells in culture (Hayashi-Nishino et al., 2009). These observations suggest that the electron-lucent membrane structures could be the seeds of the isolation membranes. In support of this suggestion, immuno-EM analysis using antibodies against Arabidopsis ATG8a, which is an autophagosomal membrane marker protein (Yoshimoto et al., 2004; Contento et al., 2005; Thompson et al., 2005), showed that ATG8 was localized on the electron-lucent membrane structures and gold particles labeled with anti-ATG8a antibodies are indicated by the black arrow (C) and white arrows or arrowheads (D), respectively. Ch, chloroplast; IM, seed of isolation membrane; Mt, mitochondrion; Per, peroxisome; Per-ED, electron-dense region in peroxisome; Vac, vacuole. Scale bars: 500 nm.
Arabidopsis thaliana ecotype Columbia was used in this study. The seeds of transfer (T)-DNA knockout mutants of ATG2, ATG5, ATG7, ATG9 and ATG10 (arg2-1 (SALK_076727), arg5-1 (SAIL_129B07), arg7-2 (GABI_655B06), arg9-3 (SALK_130796) and arg10-1 (SALK_084434), respectively) were obtained from either the Nottingham Arabidopsis Resource Center or GABI-Kat. For high-CO₂ conditions, plants were grown hydroponically under 2000 ppm CO₂ adjusted in an open gas-exchange system including phytochambers at 22°C with 65–80% relative humidity and a 12-h photoperiod.

Fluorescence microscopy

To visualize peroxisomes in arg mutants, Arabidopsis carrying a transgene encoding green fluorescent protein (GFP) fused with a peroxisomal targeting signal (GFP-PTS1) (Mano et al., 2002) was crossed to atg2, atg5 and atg7 mutants. To obtain NahG atg5 mutants expressing GFP-PTS1, atg5 GFP–PTS1 was crossed with NahG atg5 plants. GFP fusion proteins were visualized using a Zeiss LSM 510 confocal laser scanning microscope (Zeiss, Jena, Germany) as described previously (Yoshimoto et al., 2004). To visualize mitochondria and vacuolar membranes, Arabidopsis leaves were stained with MitoTracker Green (MTG; Invitrogen, Carlsbad, CA) and FM 4-64 (Invitrogen), respectively, then observed by confocal microscopy with a 488 nm laser for MTG observation (emission 495–535 nm) and a 514 nm laser for FM 4-64 observation (emission 615–645 nm). Real-time movies were taken with a fluorescence microscope (BX51; Olympus, Tokyo, Japan) equipped with a CCD camera (DP72; Olympus).

Immunoblotting analysis

Four-week-old plants were used for immunoblotting analyses. Plant total protein samples were prepared as described previously (Yoshimoto et al., 2004), except in the case of immunoblotting analysis using the pellet fraction (Fig. 2D), for which protein extracts homogenized with 100 mM Tris-HCl pH 8.0, 20% glycerol and 30 mM dithiothreitol (DTT) were centrifuged at 17,750 g for 15 min, and the resultant pellet fraction was used. Aliquots of 1 or 5 µg of protein were subjected to immunoblotting analysis using anti-catalase (Yamaguchi and Nishimura, 1984), anti-glycophore oxidase (GO; Tsugeki et al., 1993) or anti-hydroxypyruvate reductase (HPR; Mano et al., 1997), anti-peroxisomal membrane protein 14 (PEX14), anti-ADP ribosylation factor 1 (Arf1), anti-cytochrome c oxidase subunit 2 (COXII), anti-sterol methyltransferase 1 (SMT1) and anti-D1 protein of photosystem II (PsbA) antibodies (Agrisera, Vinnås, Sweden).

Electron microscopy

Non-senescent leaves from 4-week-old Arabidopsis plants grown under nutrient-sufficient conditions were used. Transmission electron microscopy (TEM) (Fig. 2A; Fig. 3C) was performed according to a previous study (Toyooka et al., 2000) with some modifications. The ultrathin sections were examined by TEM (JEM-1400; JEOL Ltd, Tokyo, Japan) at 80 kV. Immunoelectron microscopy using anti-catalase antibody (Yamaguchi and Nishimura, 1984) (Fig. 2C) was performed as described previously (Nishimura et al., 1993), except for the antibody dilution (1:5000). Immunoelectron microscopy using anti-ATG8a antibody (ab77003; Abcam, Cambridge, UK) (Fig. 3D) or anti-ubiquitin 11 (UBQ11) antibody (AS08 307; Agrisera, Vinnås, Sweden) (data not shown) was performed according to a previous study (Toyooka et al., 2009), with some modifications. Arabidopsis leaves were frozen in a high-pressure freezing machine (Leica EM PACT; Leica Microsystems GmbH). The sections were labeled with antibodies (1:10) in Tris-buffered saline (TBS) at 4°C overnight.

Catalase activity measurements

Leaf samples were taken from approximately the same position of the respective leaf and were homogenized on ice in a buffer consisting of 100 mM Tris-HCl pH 8.0, 20% glycerol and 30 mM DTT. The homogenates were centrifuged at 17,750 g for 15 min to generate supernatants and pellets. The resulting supernatant samples and pellet samples that were rinsed twice with protein extraction buffer were subjected to measurement of catalase activities spectrophotometrically, by monitoring the changes of H₂O₂ at OD₄₅₀ at room temperature as described previously (Durner and Klessig, 1996). The catalase activities in soluble and insoluble fractions were normalized to the protein levels of catalase, which were determined by immunoblotting analysis using anti-catalase antibodies.

Comparison of redox state within peroxisomes

To compare the redox state within peroxisomes between wild type and atg5, we generated transgenic wild-type and atg5 plants expressing redox-sensitive GFP (roGFP2) fused with PTS1 under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The leaves were observed by confocal microscopy. roGFP2 was exited with 405 and 488 nm lasers and the fluorescence was measured with a band pass filter.

MATERIALS AND METHODS

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of 505–530 nm. Then ratiometric images of 405:488 were generated as described previously (Meyer et al., 2007).

Construction of the estradiol-inducible complementation system

The ATG5 coding region (nucleotides 1–1014) was amplified by PCR from full-length cDNA using the primers 5′-TCCCCGGGATG-GGGGAAGAGGCCC3′ and 5′-GCTCTAGATCCCTTTAGGAG-GCTTTT-3′. The resulting PCR product was inserted into the EcoRV site of the pBlueScript II vector and the resulting plasmid was designated as pH52. To construct a plasmid for generating stable transformants expressing ATG5 cDNA driven by the estradiol-inducible promoter, pH52 was digested with Apal and Sphl and was ligated into the same sites of pE88 (Zuo et al., 2006). The construct was verified by sequencing and was introduced into atg5-1 Arabidopsis plants by the floral-dip method of in planta Agrobacterium tumefaciens (2007). Hierarchy of Atg Plant J.

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This research was funded in part by a research fellowship from the RIKEN Special Postdoctoral Researchers Program [grant number 19-062 to K.Y.]; by Grant-in-Aid for Scientific Research (B) [grant number 2270049 to K.Y.]; and by the Institut National de la Recherche Agronomique (INRA) Package Program from INRA, France. Deposited in PMC for immediate release.

http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.139709/-/DC1

Author contributions

K.Y. designed the research. M.K., M.S. and K.T. performed electron microscopic observations. K.Y. performed other experiments. K.Y., M.S., M.K., O.M.S., K.T.-K., M.S., M.N. and Y.O. analyzed the data and contributed to data interpretation and the preparation of the manuscript. K.Y. and Y.O. wrote the manuscript.

Acknowledgements

The authors would like to thank Ivanaaska for critical reading of the manuscript. The pE88 plasmid was a kind gift from Nam-Hai Chu of Rockefeller University. We also thank Mayumi Wakazaki of RIKEN for technical assistance on the EM analyses and Kazuo Tsugane of the National Institute for Basic Biology for expert care of our plants in the high-C02 experiment. The high-C02 experiment was supported by the Japan Advanced Plant Science Network.

Competing interests

The authors declare no competing interests.
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