Assessment and Optimization of Autophagy Monitoring Methods in Arabidopsis Roots Indicate Direct Fusion of Autophagosomes with Vacuoles

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Autophagy is a degradation pathway that recycles cell materials upon encountering stress conditions or during specific developmental processes. To better understand the physiological roles of autophagy, proper monitoring methods are very important. In mammals and yeast, monitoring of autophagy is often performed with a green fluorescent protein (GFP)–ATG8 fusion protein or with acidotropic dyes such as monodansylcadaverine (MDC) and LysoTracker Red (LTR). To evaluate these monitoring methods, here we examined these systems by inducing autophagy in Arabidopsis thaliana roots as a model for monitoring autophagy in planta. Under carbon- and nitrogen-starved conditions, the number and size of vesicles labeled by GFP–ATG8 was increased for several hours and then gradually decreased to a level higher than that observed before the start of the experiment. We also observed the disappearance of GFP–ATG8-labeled vesicles after treatment with wortmannin, a phosphatidylinositol 3-kinase inhibitor known as an autophagy inhibitor, showing that the GFP–ATG8 transgenic line constitutes an excellent method for monitoring autophagy. These data were compared with plants stained with MDC and LTR. There was no appreciable MDC/LTR staining of small organelles in the root under the induction of autophagy. Some vesicles were eventually observed in the root tip only, but co-localization experiments, as well as experiments with autophagy-deficient atg mutants, provided the evidence that these structures were located in the vacuole and were not manifestly autophagosomes and/or autolysosomes. Extreme caution should therefore be used when monitoring autophagy with the aid of MDC/LTR. Additionally, our observations strongly suggest that autophagosomes fuse directly to vacuoles in Arabidopsis roots.

Keywords: Acidotropic dye • Arabidopsis thaliana • ATG8 • Autophagosome • Autophagy • Monitoring.

Abbreviations: ATG, autophagy-related; E-64d, [[L-3-trans-ethoxycarbonyloxirane-2-carbonyl]-L-leucine (3-methylbutyl)amide; GFP, green fluorescent protein; LTR, LysoTracker Red; 3-MA, 3-methyladenine; MDC, monodansylcadaverine; PAS, pre-autophagosomal structure; PI3K, phosphatidylinositol 3-kinase; RNAi, RNA interference; SAV, senescence-associated vacuole; TOR, target of rapamycin.

Introduction

Macroutaphagy (referred to hereafter as autophagy) is an evolutionarily conserved intracellular process in eukaryotes in which components of cells, whether the cytosol or organelles, are degraded in the lysosome for mammals or in the vacuole for yeast and plants to provide raw materials and energy, and also to eliminate damaged or toxic components (Klionsky 2007). Autophagy occurs with the formation first of a pre-autophagosomal structure (PAS) and then of a phagophore, which forms around the components to be degraded. Once the structure closes around these components, it is termed an autophagosome, which is a double-membrane structure. The autophagosome is transported toward the lytic compartment of the cell, and the outer membrane of the autophagosome then fuses to the vacuolar and/or lysosomal membrane to release the inner membrane structure and its cargo, the autophagic body, into the lumen of the lytic organelle for degradation (Mizushima, 2007). The protein systems involved in the autophagic process were initially discovered in yeast (Xie and Klionsky 2007). Most autophagy-related (ATG) genes are well conserved throughout evolution and their homologs were found to exist in mammals and plants (Reumann et al. 2010, Tanida 2011). In general, autophagy is a very important process for living organisms: in plants, it is involved in the innate immune responses (Liu et al. 2005, Hofius et al. 2009) and during senescence (Yoshimoto et al. 2009), not to mention survival under nutrient-starved...

The autophagy machinery functions in a specific way (Yoshimoto 2012): under nutrient-starved conditions, kinase activity of target of rapamycin (TOR) is inhibited, and thus ATG1 and ATG13 assemble, forming an ATG1–ATG13 kinase complex, which may act on a possible ATG9-mediated delivery of lipids to the PAS via an ATG9 system. The addition of phosphatidylinositol-3 phosphate to the PAS may allow ATG9 to add lipids to the PAS and eventually form the phagophore. Then there is an ATG12–ATG5/ATG16-mediated lipidation of ATG8 with phosphatidylethanolamine, a lipid component of the membrane of the autophagosome (Chung et al. 2010). This makes ATG8 a particularly interesting protein, since it is anchored to the autophagosomal membrane and would constitute a good marker for the observation of autophagosome movements. In fact, many studies have been carried out using the green fluorescent protein (GFP)–ATG8 fusion protein in yeast and mammals, for example in order to study autophagosome formation in yeast (Xie et al. 2008) or to observe the autophagic response to starvation treatments in mice using a GFP–LC3 (equivalent to GFP–ATG8 in yeast) transgenic line (Mizushima et al. 2004). Thus it is now a consensus that GFP–ATG8 constructs are a very reliable way of monitoring autophagy. This monitoring method has been employed in plants (Yoshimoto et al. 2004, Contenko et al. 2005, Thompson et al. 2005).

Autophagy has also been observed with the aid of acidotropic fluorescent dyes such as monodansylcadaverine (MDC) (Contenko et al. 2005), sometimes coupled with an inhibitor of different autophagic processes such as 3-methyladenine (3-MA)/wortmannin [a phosphatidylinositol-3 kinase (PI3K) inhibitor or E-64d {{[1-3-trans-ethoxycarbonyloxirane-2-carbonyl]-l-leucine (3-methylbutyl) amide; a cysteine protease inhibitor}] (Takatsuka et al. 2011). These fluorescent dyes are known for staining acidic compartments (Takatsuka et al. 2004, Inoue et al. 2006). The plant autophagic processes have also been used in experiments of plant autophagy, notably in the observation of autolysosomes in tobacco leaf cells treated with E-64d (Liu et al. 2005). The plant autophagic processes have also been observed in tobacco BY-2 cells with a fluorescent endocytosis marker in combination with a cysteine protease inhibitor, leading to the proposal that autophagosomes acquire hydrolytic enzymes and proton pumps to become autolysosomes before fusion with the central vacuole, and that the autolysosomes play a major role in the degradation of the enclosed cytoplasm (Takatsuka et al. 2011).

There seems to be the underlying hypothesis that the methods of observing autophagy cited above are specific and reliable. That may not always be the case, notably when discussing the acidity of autophagosomes and therefore their capacity to be marked by acidotropic dyes. This is why it is interesting to perform a comparison of the methods used for monitoring autophagy, with a special emphasis on the specificity of each one.

In this study, we carefully observed the autophagic process with different monitoring methods in planta using Arabidopsis as a model for plant autophagy. In order to induce autophagy, a combination of carbon and nitrogen starvation was used, since it is known that autophagy is induced when plants are starved of different nutrients. Then, observations and comparisons were made using a GFP–ATG8 fluorescent line as well as MDC or LysoTracker Red (LTR). A detailed observation and comparison of autophagy monitoring methods suggests that extreme caution should be used in the interpretation of some results, especially those obtained with the aid of acidotropic dyes.

**Results**

**Kinetics of autophagic activity can be monitored in planta using GFP–ATG8 transgenic Arabidopsis**

In yeast and animals, GFP–ATG8 has been used as the most reliable molecular marker for autophagosomes (Mizushima et al. 2004, Xie et al. 2008). Therefore, first of all, we used GFP–ATG8 transgenic *Arabidopsis thaliana* to monitor autophagic activity in planta. In order to test different methods for observation of autophagy, an autophagy induction method has to be employed. Since there were already reports of the importance of autophagy during nitrogen starvation, as well as carbon starvation (Izumi et al. 2010, Guiboileau et al. 2013), we chose to combine the two starvation conditions for a better autophagic response.

Under nitrogen starvation in combination with carbon starvation conditions, the induction of autophagy was observed in a wild-type GFP–ATG8 *A. thaliana* line as well as a TOR-RNA interference (RNAi) line expressing GFP–ATG8 (Fig. 1). Under nutrient-rich conditions, several small dot structures with a diameter <0.5 μm labeled by GFP–ATG8 were visible in the roots (Fig. 1A; inset at 0 h). Then, after transfer to starved media, the number and size of the dot structures started to increase immediately and increased steadily over several hours (Fig. 1A, D, E). After about 4 h of starvation incubation, the ring-shaped structures were more abundant and larger in size (Fig. 1A, inset at 4 h). The number of dot structures peaked about 2–3 h after the beginning of starvation and decreased thereafter, but still to a level higher than the original number of the structures. The small dot structures observed are probably PASs, which will then become ring-shaped autophagosomes under autophagy-inducing conditions. We tracked the percentage of PASs (diameter <0.5 μm) relative to the percentage of autophagosomes present (diameter >1 μm) (Fig. 1E). Over the passage of 24 h, the percentage of autophagosomes rapidly increases while the percentage of PASs, which are predominant in the beginning, steadily decreases. Autophagosomes are most abundant at 4 h after the beginning of starvation, representing 58% of the total population of the structures, and then their
percentage decreases again. After 24 h, PASs and autophagosomes are present equally in the cell.

Parallel with the GFP–ATG8 line, we observed a TOR-RNAi line since there was a study showing that knockdown of TOR in Arabidopsis resulted in constitutive autophagy (Liu and Bassham 2010). As observed in that report, even under nutrient-rich conditions, the basal level of the number of the dot structures labeled by GFP–ATG8 was much higher in the

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**Fig. 1** Monitoring of the induction of autophagy by GFP–ATG8 fusion protein in Arabidopsis roots. Seven-day-old seedlings of GFP–ATG8 transgenic plants grown vertically under nutrient-rich conditions were transferred to solid media lacking carbon and nitrogen and then the roots were observed by confocal and conventional light microscopy. (A) Behavior of autophagosomal structures revealed by GFP–ATG8 during 24 h of carbon and nitrogen starvation in Arabidopsis wild-type roots. (B) Comparison of the number of autophagosomal structures in wild-type and TOR-RNAi roots expressing GFP–ATG8 during carbon and nitrogen starvation. (C) DIC images of wild-type and TOR-RNAi roots treated with concanamycin A under carbon- and nitrogen-starved conditions. (D) Quantification of the number of autophagosomal structures observed in wild-type (blue) and TOR-RNAi (red) roots under carbon- and nitrogen-starved conditions. The number of autophagosomal structures per image was counted at each time point and the averages were determined and converted to a number per μm² (n = 5, at least). (E) Proportion of autophagosomes (>1.0 μm, green) and pre-autophagosomal structures (PASs; <0.5 μm, orange) present over time in wild-type roots under carbon- and nitrogen-starved conditions. Scale bars = 25 μm for (A), 5 μm in insets, 50 μm for (B) and (C).
TOR-RNAi line than in the wild-type line (Fig. 1B, D). Interestingly, the number was further increased by the starvation treatment and gradually decreased with kinetics similar to those of the wild-type line (Fig. 1B, D). The increase in the number of dot structures observed suggests that autophagy is more induced in the TOR-RNAi line, but there is still a possibility that in this line, the fusion of autophagosomes with the vacuole might be inhibited, leading to an accumulation of autophagosomes in the cytoplasm. In order to exclude this possibility, a concanamycin A treatment experiment was performed (Fig. 1C). When plant cells are treated with the V-ATPase inhibitor, concanamycin A, autophagic bodies can be detected inside the vacuolar lumen as small randomly moving vesicles, thus allowing us to monitor autophagic flow (Yoshimoto et al., 2004). After treatment with concanamycin A for 3.5 h, wild-type roots slowly started to accumulate autophagic bodies in the vacuolar lumen, and in the TOR-RNAi line autophagic bodies were already highly accumulated even at this early time point. These results indicate that autophagy is already activated in the TOR-RNAi line under nutrient-rich conditions and that it is further induced by nutrient-starved conditions.

To confirm the autophagic nature of the GFP–ATG8 fluorescent structures further, an autophagy inhibition experiment using wortmannin was conducted (Fig. 2). Wortmannin has been widely used as an autophagy inhibitor based on its inhibitory effect on class III PI3K activity, which is known to be essential for induction of autophagy (Blommaart et al. 1997). After transfer to carbon- and nitrogen-starved media, the number of autophagosomes was increased (Fig. 2B, upper panel). However, when the plants were simultaneously treated with wortmannin, the root cells only presented extremely few very small structures like PASs (Fig. 2B, lower left panel, white arrowheads). After wash-out of wortmannin, an increased number of dot- or ring-shaped structures was again detected (Fig. 2B, lower right panel). Parallel to this and in the same experimental conditions, concanamycin A treatments induced accumulation of autophagic bodies in the vacuoles (Fig. 2D, upper panel), while simultaneous addition of wortmannin inhibited the accumulation of autophagic bodies (Fig. 2D, lower panel), proving that the GFP–ATG8 fluorescent dots are actually autophagy related and permitting the use of this line in order to monitor autophagy in planta.

**Fig. 2** Autophagy is inhibited by wortmannin treatment in Arabidopsis roots. (A, C) Schematic diagrams showing wortmannin treatment experiments. (A) Seven-day-old seedlings of GFP–ATG8 transgenic plants grown vertically on nutrient-rich (NR) solid media were transferred to nutrient-rich liquid media with (NR + wort) or without (NR) wortmannin (0 h) and incubated for 2 h. Then, the seedlings were transferred to liquid media lacking carbon and nitrogen with (–C–N + wort) or without (–C–N) wortmannin, respectively, and the roots were observed by confocal microscopy after 3 h incubation (5 h; B, left panels). Wortmannin-treated roots were again observed after wash-out of wortmannin (7 h; B, right panel). (C) Roots incubated in NR or NR + wort for 2 h were transferred to –C–N with only concanamycin A (–C–N + con A) or with both concanamycin A and wortmannin (–C–N + con A + wort), respectively. After 5 h incubation (7 h), roots were observed by conventional light microscopy. DIC images are shown in (D). White arrowheads in (B) indicate small dot structures, which are putative pre-autophagosomal structures. The high level of fluorescence observed at the interstices of root cells in (B) indicates the area of cytoplasm, which is enlarged, probably because the size of the central vacuoles is reduced after the action of wortmannin. Scale bars = 50 µm in (B), 20 µm in (D), 5 µm in insets.
Monodansylcadaverine does not label autophagosomes in Arabidopsis roots

There is a report showing co-localization of MDC-stained vesicles and GFP–ATG8 fluorescent autophagosomes in Arabidopsis (Contento et al. 2005). However it was observed only in protoplasts of suspension-cultured cells and thus it was not known if this is the case in planta. Therefore we performed MDC staining using Arabidopsis roots to check if MDC labels autophagosomes in planta. Here, MDC was used as a possible means to monitor autophagy in conjunction with GFP–ATG8 fluorescent lines. To observe MDC staining in *A. thaliana* roots, an experiment was conducted on the TOR-RNAi line under the same conditions as described above (Fig. 3). Over a period of 4 h starvation, during which we confirmed that autophagosomes were increased as shown in Fig. 1, MDC-positive vesicles were not detected above the root elongation zone (referred to

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**Fig. 3** Monitoring autophagy with MDC staining. Roots were grown vertically on nutrient-rich media for 7 d, then transferred to solid media lacking carbon and nitrogen and observed under a fluorescence microscope. (A) MDC-stained TOR-RNAi roots during induction of autophagy over 4 h by carbon + nitrogen starvation. (B) MDC-stained GFP–ATG8 transgenic roots after approximately 4 h of induction of autophagy by carbon + nitrogen starvation. (C) MDC staining in a GFP–ATG8 transgenic root tip after approximately 4 h of starvation. (D) MDC-stained root tips of wild-type and atg5 after approximately 4 h of starvation. White arrowheads indicate autophagosomal structures labeled with GFP–ATG8. Blue arrowheads indicate MDC-labeled structures. Scale bars = 20 μm, 5 μm in insets.
hereafter as the root), even in this TOR-RNAi line which has higher autophagic activity (Fig. 3A). Then, co-localization experiments with GFP–ATG8 were performed in the wild-type line because the expression level of GFP–ATG8 in the TOR-RNAi line was too low for comparison. The results showed that there was never an overlap of MDC staining and GFP–ATG8 fluorescence in the root (Fig. 3B: Supplementary Fig. S1).

In root tips, on the other hand, some MDC-positive vesicles were occasionally detected (Fig. 3C, D). The MDC-stained vesicles appeared larger than the GFP–ATG8 fluorescent vesicles seen and were moving erratically in the vacuole. These vesicles are therefore not autophagosomes, which should be located in the cytoplasm. We also occasionally detected the same type of rapidly moving MDC-stained vesicles in atg5 and atg2 root tips (Fig. 3D: Supplementary Fig. S2A). Co-localization experiments with GFP–ATG8 in the root tips showed no overlap of GFP–ATG8 and MDC fluorescence (Fig. 3C), further confirming that MDC-stained structures are not autophagosomes. Altogether, these results indicate that MDC does not stain autophagosomes in planta.

LysoTracker Red does not specifically label autophagosomes/autolysosomes in Arabidopsis

LysoTracker has been also reported as an indicator of autophagosomes or autolysosomes especially in animals and recently also in plants (Rodriguez-Enriquez et al. 2006, Kwon et al. 2013); therefore, we checked in TOR-RNAi roots if LTR specifically labels autophagosomes/autolysosomes in planta. In order to check autophagy-related staining by LTR, we observed the situation after E-64d treatment with the central vacuole, and that the autolysosomes play a major role in the degradation process in tobacco suspension-cultured cells (Takatsuka et al. 2011). In Arabidopsis roots, concanamycin A treatment, which will provoke inhibition of vacuolar degradation, results in accumulation of autophagic bodies in the vacuolar lumen, suggesting that autophagosomes fuse directly to vacuoles. The difference in the autophagic process between tobacco and Arabidopsis suggests that different autophagic pathways exist in different plant species. Therefore, we observed the situation after E-64d treatment in A. thaliana roots with the aim of confirming the aforementioned results in planta.

We first looked at Arabidopsis transgenic roots in which vacuolar membranes can be visualized by monitoring GFP fluorescence (ProVAM3:GFP:VAM3/vam3-1) to determine if the aggregates formed after treatment with E-64d are in the vacuolar lumen or in the cytoplasm. After treatment with E-64d for 12 h, large aggregates were detected at the center of cells, surrounded by vacuolar membranes, not always close to the nucleus (Fig. 5A, left panel). This differs from the situation in BY-2 cells, suggesting that these are not aggregates of autolysosomes, but more likely to be autophagic bodies. We then performed time-lapse imaging of E-64d-treated roots at early time points in order to observe the dynamic process of aggregation (Fig. 5B). In the first 3 h of E-64d treatment, there was initially

Treatment with E-64d, a cysteine protease inhibitor, results in accumulation of autophagic bodies inside vacuoles in Arabidopsis root cells, unlike in tobacco BY-2 cells

In tobacco BY-2 cells, autophagic processes have been observed with the aid of E-64d, a cysteine protease inhibitor, coupled with both an autophagy inhibitor such as 3-MA and acidotropic dyes such as LTR or FM 4-64 (Moriyasu and Inoue 2008). In E-64d-treated BY-2 cells, small acidic compartments which seem to be autolysosomes are clustered and form aggregates around the nucleus, not in the vacuolar lumen. From these observations, it has been proposed that autophagosomes acquire hydrolytic enzymes to become autolysosomes before fusion with the central vacuole, and that the autolysosomes fuse directly to vacuoles. The difference in the autophagic process between tobacco and Arabidopsis suggests that different autophagic pathways exist in different plant species.
detection of single vesicles in the vacuole similar to the autophagic bodies detected after concanamycin A treatment (Fig. 5B, white arrows; Fig. 1C), then, after approximately 3 h of treatment, these single vesicles started to form aggregates and eventually fused into even larger aggregates. This aggregation process was not observed in atg mutants (data not shown), suggesting that they are autophagic bodies. Time-lapse imaging revealed dynamic movement of two small aggregates forming a larger one (Fig. 5C; Supplementary Movie S2). The small aggregates shown by black and white arrowheads were moving randomly at the beginning (Fig. 5C, 1–7 s), indicating that they were inside the vacuole, and the large aggregate formed became
stable (Fig. 5C, 8–10 s, black arrows). This result is consistent with a previous report showing that in E-64d-treated Arabidopsis root cells, inclusions corresponding to cytoplasm degradation intermediates accumulate in the central vacuole (Inoue et al. 2006). In A. thaliana roots, therefore, E-64d treatment causes the accumulation of autophagic bodies inside the vacuole.

**Discussion**

The GFP–ATG8 fusion is the most often used to monitor autophagy in eukaryotes. Additionally, acidotropic dyes also have their place in the observation of autophagic processes, especially in mammals (Rodriguez-Enriquez et al. 2006, Chikte et al. 2014). However, there is no report comparing different types of monitoring methods in planta. In this study, therefore, we focused on comparing these methods in order to confirm the best system to use, which is essential for understanding physiological roles of autophagy. We found numerous problems that arise with the use of acidotropic dyes, notably their non-specificity in staining autophagy-related structures as well as the fact that LTR and MDC do not stain autophagosomes in planta.

**ATG8 fused with fluorescent proteins is the best marker for monitoring autophagic processes in planta**

The observation of the GFP–ATG8 line allowed the demonstration of autophagy induction in A. thaliana by carbon and nitrogen starvation (Fig. 1). The GFP–ATG8 fluorescent TOR-RNAi line seems to be the best line to use since autophagic activity is enhanced in that line and the kinetics of autophagy induction follow that of the wild-type GFP–ATG8 line (Fig. 1). The high number of autophagosomes would permit better observation of autophagy under different environmental conditions.

With the aid of a PI3K inhibitor, wortmannin, known as an autophagy inhibitor, we confirmed that the transgenic GFP–ATG8 fluorescent line is also a good tool for monitoring autophagic processes in planta. Under the influence of wortmannin, root cells of the GFP–ATG8 line showed very few and very small dot structures (Fig. 2B). In yeast, there is a study tracking the localization of GFP–ATG8 in different autophagy mutants (Suzuki et al. 2007). The yeast *atg14* mutant, in which autophagic functions are perturbed at the level of the PI3K complex, exhibited small and less numerous GFP–ATG8 fluorescent dot structures than those of wild-type yeast, leading to the conclusion that the PI3K complex is important for targeting of ATG8.
to the PAS. Our observation is consistent with these data; therefore we concluded that the small dot structures labeled with GFP–ATG8 in the wortmannin-treated cells are PASs. After wash-out of wortmannin, the small dot structures became large and ring-shaped structures which are thought to be autophagosomes. Using this method, it is now possible to observe autophagic processes from the PAS to the beginning of autophagosome formation and also from autophagosome enclosure to fusion with vacuoles.

**Possible secondary effects arising from the use of dyes and staining non-specificity**

In this study, we indicated that acidotropic dye use is problematic for monitoring autophagy in planta. There was no co-localization of MDC/LTR and GFP–ATG8 fluorescence, conclusively proving that MDC and LTR do not in fact stain autophagosomes in Arabidopsis root cells. It is possible that, irrespective of acidity, MDC and LTR somehow stain autophagic bodies which are being degraded in the vacuolar lumen, and in that case there might be co-localization of MDC/LTR and GFP–ATG8 fluorescence. Indeed, the E-64d-induced aggregates localized inside the vacuolar lumen (Fig. 5), which are autophagic bodies, were stained with MDC/LTR although the fluorescence signal intensities were not so high (data not shown). On the other hand, the E-64d-induced aggregates did not frequently co-localize with GFP–ATG8 fluorescence, although in some cases partial co-localization was observed (data not shown). This low frequency of the co-localization is probably due to the lower integrity of autophagic bodies in E-64d-treated cells. When plant cells are treated with E-64d, fewer round-shaped single autophagic bodies are present, while aggregated autophagic bodies are abundant, suggesting that the membranes of autophagic bodies are degraded. Therefore, it seems that the inside of the autophagic bodies is exposed to the lytic conditions of the vacuolar lumen, leading to inactivation or degradation of GFP–ATG8. Accumulation of autophagic bodies does not necessarily mean high autophagic activity, because if vacuolar hydrolase activity was somehow low, autophagic bodies could be easily accumulated. Without observation of autophagosomes, we cannot distinguish whether high accumulation of autophagic bodies is caused by high autophagic activity or by a diminished efficiency of vacuolar degradation. Therefore, it is necessary to distinguish autophagosomes and autophagic bodies clearly during autophagy monitoring to interpret the autophagic process and its activity correctly.

In addition, we observed the presence of LTR- and MDC-stained vesicles even in atg5 and atg2 (Figs. 3D, 4B; Supplementary Fig. S2), meaning that LTR and MDC non-specifically stain some structures that are not autophagy related in the vacuolar lumen. It would be interesting to explore whether these MDC- and LTR-stained structures are in fact co-localized. These autophagy-independent structures observed in the vacuole could, for example, be derived from a multivesicular body pathway. Thus, it is impossible to distinguish autophagy-related cargo from the non-autophagy-related cargo in the vacuole by staining with acidotropic dyes. In fact, all the LTR and MDC fluorescence observed in the root tip was not actually consistently present even in wild-type plants. Some root tips showed little to no staining, while others were heavily stained, this occurring in the same group of plants that were subjected to the same autophagy induction and staining treatment. For LTR, often staining was observed in cells that did not have GFP fluorescence, whether from damage to the root or because of the chimeric nature of GFP–ATG8 expression in that particular root. This indicates that the dye’s action is not homogenous and depends on a factor that is as yet unknown. Therefore, it was not even possible to quantify statistically the stained structures, thus making them unreliable for in planta studies. Furthermore, Viotti et al. (2013) reported quite recently that, in meristematic cells of the A. thaliana root, small lytic vacuoles that are referred to as provacuoles are formed even in atg mutants. Therefore, the root tip area would not be a good place for monitoring autophagic structures with the aid of acidotropic dyes.

During MDC staining experiments, we also noticed that MDC has some negative effect on the autophagosomal membrane. Two hours after the beginning of autophagy induction by carbon- and nitrogen-starved media and after MDC staining, some of the GFP–ATG8 fluorescent structures observed in the root had irregular shapes (Supplementary Fig. S3): instead of observing ring-shaped autophagosomes, we observed truncated, tubular structures, suggesting a possible toxicity of MDC to the cell and the perturbation of the autophagic process. The tubular structures would be ruptured autophagosomes. In this case, it is all the more important to be cautious in the interpretation of results obtained with this staining method.

**Short-term treatment with E-64d allows us to detect dynamic movements of autophagic bodies in Arabidopsis root cells**

There is a study on the retrograde transfer of vacuolar membranes and hydrolytic enzymes to the autophagosomes of tobacco BY-2 cells; these components enable them to become acidified and be classified as autolysosomes. These autolysosomes are then apparently observed in the perinuclear region after 1 d of treatment with E-64d (Yano et al. 2004). In the same manner, another study suggests that late endosomes observed in the cytoplasm under sucrose-starved conditions in BY-2 cells after prolonged treatment with E-64d could be autolysosomes (Yamada et al. 2005). Although these reports suggested that E-64d induces vesicles containing vacuolar membranes and/or endosomal materials in the cytoplasm, here we found that E-64d induces accumulation of autophagic bodies inside the vacuolar lumen of Arabidopsis root cells (Fig. 5). This discrepancy may be explained by the differing E-64d incubation time. We only detected dynamic movements of autophagic bodies inside the vacuolar lumen after short-term (~3 h) treatment
with E-64d. Therefore, E-64d may have pleiotropic effects on vesicle trafficking depending on the incubation time. Further detailed kinetic analyses will allow us to understand these phenomena completely.

In addition to the finding that autophagic bodies are accumulated inside vacuoles after E-64d treatment, our MDC/LTR staining experiments revealed that there are no acidic small vesicles in the cytoplasm even under autophagy induction conditions (Figs. 3, 4). If autolysosomes exist and play a major role in the degradation process, the increased number of autolysosomes, which are acidic vesicles, should also be observed at the same time as the autophagosomes, but they were not detected even at the later time points of carbon and nitrogen starvation (after ~6 h), at which the number of autophagosomes started to decrease. Taken together, these results indicate that autophagosomes directly fuse to vacuoles in Arabidopsis roots, although the existence of an autolysosome pathway cannot be completely ruled out.

### Microscopic analysis and its requirements

All observations were performed using confocal or fluorescent microscopy, which include a number of constraints that should be taken into account for perfecting autophagy monitoring methods. First, microscopic analysis of root cells cannot be carried out with a low-magnification lens, since that makes it impossible to distinguish the exact size and location of the vesicles. Methods. First, microscopic analysis of root cells cannot be carried out with a low-magnification lens, since that makes it impossible to distinguish the exact size and location of the vesicles. Therefore, we recommend the use of high-magnification microscopy, which include a number of constraints that should be taken into account for perfecting autophagy monitoring methods. However, the high-magnification imaging enabled us to perform co-localization experiments as well as obtain data on the different types of movement of the vesicles observed. One of the characteristics of autophagosomal movement is that their transport in the cytoplasm follows a distinct pattern of cytoplasmic streaming which is oriented in a general direction and not at all erratic (Supplementary Movie S3). When we treated root cells with MDC/LTR for a long time (>10 min), some structures stained by the dyes were occasionally detected, but it is impossible to determine their nature since long exposure to the dyes could non-specifically affect any number of intracellular structures. From the erratic movement of the MDC/LTR-stained structures observed inside the vacuole, we can at least conclude that MDC/LTR-stained structures are definitely not autophagosomes. Therefore, we recommend the use of high-magnification lens and also to perform time-lapse imaging in order to observe the autophagic process exactly.

There are also issues surrounding the observation of MDC in confocal microscopy. The lowest wavelength for its excitation available was 405 nm and is used for DAPI (4′,6-diamidino-2-phenylindole) observations as well as observation of MDC. However, the excitation wavelength for MDC is actually closer to 335 nm and its emission spectrum peaks around 518 nm (Sabnis 2010), while GFP was excited by a 488 nm laser and its emission peaked around 509 nm. This makes simultaneous observation of the two impossible, as well as making confocal imaging of MDC fluorescence undesirable because of the shift between the known excitation wavelength and the laser used. Therefore, proper microscopy should be carefully selected for monitoring autophagy depending on the dyes used; otherwise the data could be misinterpreted.

### Materials and Methods

#### Plant materials

*Arabidopsis thaliana* ecotype Columbia was used in this study. The seeds of T-DNA knockout mutants of ATG5 (atg5-1, SAIL_129B07) and ATG2 (atg2-1, SALK_076727) were obtained from Nottingham Arabidopsis Resource Center (Thompson et al. 2005, Inoue et al. 2006). The GFP–ATG8f, TOR-RNAi, and ProVAM3:GFPPAM3/vam3-1 lines used in this study were provided by Professor Gad Galili (Sláviková et al. 2005), Dr. Christian Meyer (Deprost et al. 2007) and Dr. Chieko Saito (Uemura et al. 2010, Saito et al. 2011), respectively. For generation of the TOR-RNAi line expressing GFP–ATG8e, the coding sequence of AtATG8e lacking a stop codon was first amplified with 5′-AAAAAGCAGGCTCTAAAATGAATAAAGGAAGCATTCTT-3′ and 5′-AGAAACGCTGGTGCATTGAAGAAGGACCGAATGTT-3′ primers. The PCR product was cloned in pDONR207 by a BP reaction. An LR reaction cloned the chimeric gene in pGWB6 (GATEWAY binary vector, Invitrogen) containing GFP 5′ of the gene. The construct was introduced into the *Agrobacterium tumefaciens* C58PMP90 strain by electroporation, and then into the A. thaliana TOR-RNAi line by *Agrobacterium*-mediated transformation using the floral dip method. Homozygous transformants selected by antibiotic resistance were used.

#### Conditions for plant growth and induction of autophagy

Plants were grown vertically on solid MGRL media (0.4% Gelrite, Fujiwara et al. 1992) supplemented with 4 mM KNO₃ and 1% sucrose [nutrient-rich (NR) media] at 22 °C with 16 h light/8 h dark cycles for 6–8 d before observation. For nutrient starvation, they were transferred to nitrogen- and sucrose-depleted MGRL solid media (–C–N). In –C–N experiments, the plants were kept in the dark to reduce sucrose production by photosynthesis.

#### Data analysis used

In order to quantify the number of autophagic structures observed in **Fig. 1**, the structures were counted using several (at least five) z-stack merged images. PASs were defined as structures whose diameter was <0.5 μm, while autophagosomes were defined as having a diameter >1 μm. This was done in order to separate the two groups clearly and account for image dispersion occurring when using confocal imagery.
based on the average structure sizes observed. The number of structures was counted per μm². All data analysis was performed using Imagej and Excel.

**Autophagic process inhibition treatments**

For wortmannin treatment, 1-week old seedlings grown on NR media were transferred to NR or –C–N liquid media containing 5 μM wortmannin prepared from 5 mM stock dissolved in dimethylsulfoxide (DMSO). For E-64d treatment, 1-week old seedlings were incubated in the dark in MES buffer containing 10 μM E-64d dissolved in DMSO for the period indicated. Concanamycin A treatment was done using a 1 μM concentration as previously described (Yoshimoto et al. 2004).

**MDC and LTR staining**

Plants were stained with MDC at 50 μM final concentration for 10 min, and then washed three times with phosphate-buffered saline (PBS) as previously described (Xiong et al. 2006). LTR staining was done with 1 μM LTR in 10 mM HEPES-Na (pH 7.5) as previously described (Moriyasu et al. 2003), then washed once with PBS and observed. The staining solutions were kept in the dark.

**Microscopy conditions**

Confocal microscopy was done on an Inverted LEICA SP2 AOBs with a 488 nm argon laser for GFP observation (BP 495–530 nm) and a 543 nm laser for LTR observation (BP 570–630 nm). The objective used was an HCX PL APO lbd. BL × 63.0 1.2W water objective. For fluorescence microscopy, an upright Zeiss AXIO Imager Z was used with a DAPI filter (Filter set 49: excitation BP 300–380, beam splitter: 395, emission BP 420–470) for MDC observation and a GFP filter (AHF-495–530 Sp Green: excitation 484–504, beam splitter: 506, emission BP 420–470) for MDC observation and a GFP filter (AHF-300–380, beam splitter: 395, emission BP 420–470) for DAPI observation. For DIC imagery, a Zeiss Axio Imager (Zeiss #420762-9800). For DIC imagery, a Zeiss Axio Imager

**Disclosures**

The authors have no conflicts of interest to declare.

**References**


