In vivo imaging and quantitative monitoring of autophagic flux in tobacco BY-2 cells

Shigeru Hanamata,1,3 Takamitsu Kurusu,1,2,4 Masaaki Okada,1 Akiko Suda,1 Koki Kawamura,1 Emi Tsukada1 and Kazuyuki Kuchitsu1,2,*

1Department of Applied Biological Science; Tokyo University of Science; Noda, Chiba, Japan; 2Research Institute for Science and Technology; Tokyo University of Science; Noda, Chiba, Japan; 3Current affiliation: School of Bioscience and Biotechnology; Tokyo University of Technology; Hachioji, Tokyo, Japan

*These authors contributed equally to this work.

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Abbreviations: BY-2, bright yellow-2; BY-YA8, transgenic tobacco BY-2 cell line (BY-YA8) stably expressing a YFP-NtATG8a fusion protein; BY-HGA8ΔG, transgenic BY-2 cell line expressing an HA-tagged GFP fused with a C-terminal glycine deletion mutant of the NtATG8a protein; BY-HRYA8, transgenic BY-2 cell lines expressing HA-mRFP-YFP-NtATG8a fusion protein; HRYA8ΔG, transgenic BY-2 cell lines expressing HA-mRFP-YFP-NtATG8aΔG fusion protein; CA, concanamycin A; CLSM, confocal laser-scanning microscopy; DIC, differential interference contrast; LC3, microtubule-associated protein light chain 3; GFP, green fluorescent protein; mRFP, monomeric red fluorescent protein; 3-MA, 3-methyladenine; RFP, red fluorescent protein; YFP, yellow colored variant of GFP; PI3K, phosphoinositide 3-kinase; BTH, benzo-(1,2,3)-thiadiazole-7-carboxylic acid S-methyl ester

Introduction

Macroautophagy (hereafter referred to as autophagy) is an evolutionarily conserved system for degradation of intracellular components through the vacuole/lysosome, which has been shown to play essential roles in the growth, development and survival of eukaryotic cells. Autophagy is a process that starts with the formation of a cup-shaped phagophore in the cytoplasm that becomes a double-membrane structure, called the autophagosome, and engulfs long-lived proteins and organelles.1-3 The autophagosome subsequently fuses with the vacuole/lysosome, and the inner membrane, the autophagic body, is degraded by hydrolytic enzymes.1-3 These changes are called autophagic flux.4 Various methods have been used to analyze autophagic flux in plants.5 Though transmission electron microscopy is effective...
to directly observe autophagy-related intracellular structures,\(^5_7\) it is not suitable for characterization of their in vivo dynamics. A cysteine protease inhibitor E-64 and a vacuolar H\(^+\)-ATPase inhibitor concanamycin A (CA) allow light microscopic observation of intracellular accumulation of autolysosomes and autophagic bodies, respectively.\(^8\) However, this technique is not suitable for real-time imaging or quantitative monitoring because accumulation of these visible structures takes a long time. Concanamycin A may also affect the activity of normal autophagic flux.\(^10\) Another method uses monodansylcadaverine (MDC) and Lysotracker as a marker probe for acidic compartments to detect autolysosome-like structures.\(^11,12\) However, these methods are not specific for autophagy,\(^13\) and therefore need to be combined with other techniques.\(^3\)

Over 30 known autophagy-related genes (ATGs) have been identified in yeast, many of which are conserved in most eukaryotes including mammals and plants.\(^14,15\) Formation of the autophagosomes requires two ubiquitin conjugation-like reactions with ATG12 and ATG8.\(^2\) The C-terminal glycine-residue of ATG8 has been shown to be essential for the conjugation reaction and autophagosome formation.\(^9,14\) In animal cells, electrophoretic detection of the lipidation of microtubule-associated protein light chain 3 (LC3), a mammalian homolog of ATG8, has been established as a quantitative marker for autophagy.\(^4\) However, this method has not been established in plant cells.

ATG8 fused with the green fluorescent protein (GFP) have enabled the observation of autophagosomes in living cells.\(^9,11,16,17\) The GFP fluorescence can be observed in punctate and ring-shaped structures, which increase under nutrient starvation or stress condition.\(^16,18\) These structures are transported from the cytoplasm to the vacuole,\(^9,16\) and transported GFP-ATG8 protein is processed in vacuolar lumens.\(^9\) The processing of GFP-ATG8 protein has been detected by western blot analysis,\(^19,20\) but this method is not suitable for non-invasive real-time detection. The tandemly fused fluorescent proteins have been used for monitoring the mitophagy and the autophagosome maturation in yeast and animals.\(^21,22\) However, in plant cells, such approaches have not been applied to monitor autophagy, and simple methods for quantification of autophagic flux remain to be developed.

Tobacco BY-2 cells are superior for analyzing the intracellular localization and dynamics of proteins and organelles, as well as cell cycle-related phenomena.\(^23\) Four isoforms of ATG8 genes named NtAtg8a, b, c, d have been found from the EST database (http://mrg.psc.riken.go.jp/strc/index.htm). NtAtg8a mRNA has been suggested to be expressed in lag, log and stationary phase cells. NtAtg8b-d have also been obtained from a cDNA library generated from cells treated with several plant hormones or under sucrose starvation conditions.\(^24\)

We here established a non-invasive monitoring system for autophagic flux in tobacco BY-2 cells expressing NtATG8a fused to a variety of fluorescent tags. Simultaneous in vivo imaging of the autophagosome formation, decrease in cytosolic ATG8 and accumulation of ATG8 in the vacuole in living cells allowed characterization of in vivo dynamics of autophagic flux. Furthermore, we introduce a novel simple method to monitor the autophagic activity in living cells by ratiometric fluorescence measurement. These in vivo quantitative monitoring systems of autophagy should provide a powerful tool for characterizing autophagy in plant cells.

**Results and Discussion**

**In vivo imaging of autophagic flux.** To visualize the dynamics of the autophagic flux in tobacco BY-2 cells, we generated a transgenic tobacco BY-2 cell line (BY-YA8) stably expressing a YFP-NtATG8a fusion protein\(^20\) under the control of the cauliflower mosaic virus 35S promoter. Under normal growth conditions, YFP fluorescence was detected in the cytoplasm and nucleoplasm of 3-d-old cultured BY-YA8 cells (Fig. 1A, Control). A few punctate signals of YFP-NtATG8a were observed in the cytoplasm. When the BY-YA8 cells were transferred to sucrose-free medium, an increase of punctate signals (Fig. 1A, Starvation) was observed. It reached a plateau at 2–3 h and did not change until 6 h under sucrose-starved conditions (Fig. 1B).

The phosphoinositide 3-kinase (PI3K) plays an essential role in the formation of the autophagosome.\(^25\) A PI3K inhibitor, 3-methyladenine (3-MA), has been shown to inhibit autophagy in many eukaryotic cells including the BY-2 cells.\(^26\) To confirm if the punctate signals derived from YFP-NtATG8a corresponds to the autophagosome, we tested the effects of several PI3K inhibitors. As shown in Figure 1C, the presence of 3-MA or wortmannin in culture media for 3 h clearly inhibited the number of punctate signals compared with the control (Fig. 1C and D), suggesting that the punctate signals are the autophagosomes.

The C-terminal glycine residue of ATG8 is essential for the association with the autophagosome in all eukaryotic cells,\(^27\) and deletion or point mutation of this glycine residue in the ATG8 protein has been used as a negative control of autophagic flux.\(^28\) The C-terminal glycine residue is conserved in tobacco ATG8 homologs.\(^24\) Thus we also established a transgenic BY-2 cell line (BY-HGA8ΔG) expressing an HA-tagged GFP fused with a C-terminal glycine deletion mutant of the NtATG8a protein (Fig. 2A, NtATG8aΔG). In BY-HGA8ΔG cells, the GFP signal was detected in the cytoplasm and nucleoplasm similarly to the BY-YA8 cells (Fig. 2B, Control). However, when the cells were transferred to sucrose-free medium for 5 h, in contrast to the BY-YA8 cells, no punctate signals were observed (Fig. 2B, Starvation). This indicates that the C-terminal glycine residue of NtATG8a is also essential for the association with the autophagosome in tobacco, and the punctate structures are indeed autophagosomes.

A vacuolar H\(^+\)-ATPase inhibitor, CA, induces alkalinization of the vacuole and accumulation of vesicular structures, which are assumed to be autophagic bodies.\(^3,17,29\) Under sucrose-starved conditions, we also observed similar vesicular structures in the vacuole after CA treatment in both BY-YA8 (Fig. 3A, DIC) and BY-HGA8ΔG cells (Fig. 3B, DIC), suggesting that these vesicular structures observed in BY-2 cells correspond to autophagic bodies.
To monitor the autophagosome transport from the cytoplasm to the vacuole, we treated the BY-YA8 cells with CA. An accumulation of YFP fluorescence was detected in the vacuole after 24 h in sucrose-free conditions (Fig. 3A). In contrast, no accumulation was detected in BY-HGAΔG cells (Fig. 3B), indicating that the YFP-NtATG8a fusion protein as an autophagic
marker was transported selectively from the cytoplasm to the vacuole in BY-2 cells. Taking these results together, the BY-YA8 cell line allows us to monitor the dynamics of autophagic flux in living plant cells.

The fluorescence intensity of the YFP-ATG8 protein as a suitable marker for autophagic flux. Though the in vivo imaging of BY-YA8 cells is useful to analyze the dynamics of autophagy, precise and convenient quantification (or counting) of fluorescent punctate signals in the cytoplasm of intact plant cells using fluorescence microscopy is not easy. To investigate the fluorescence intensity of YFP-NtATG8a as a marker for autophagic flux in plant cells, we analyzed the changes of fluorescence intensity in BY-YA8 cells. In sucrose-rich conditions, the YFP fluorescence showed almost no change (Fig. 4A and C, Control). In contrast, the YFP signal gradually decreased in a time-dependent manner after induction of autophagy by sucrose starvation (Fig. 4A and C, Starvation), and the reduction of YFP fluorescence was significantly inhibited by a PI3K inhibitor, 3-MA (Fig. 4A and C, St + 3-MA). In contrast, in BY-HGA8ΔG cells, the intracellular GFP signal was not affected by sucrose starvation for 24 h (Fig. 4B and D, Starvation). These results are consistent with those with transgenic animal cells expressing GFP-LC3\(^{30}\) and suggest that YFP-NtATG8a is selectively degraded by autophagy, which can be quantitatively analyzed as a marker for autophagic flux.

We next quantitatively monitored the accumulation of YFP fluorescence in the vacuole in the presence of CA that inhibits vacuolar degradation or quenching of YFP. As discussed above, the fluorescence of YFP-NtATG8a in the cytoplasm and nucleoplasm decreased under sucrose starvation [Fig. 5A, CA (-)]. At the same time, in the absence of CA, the YFP fluorescence in the vacuole showed almost no change under sucrose-free conditions [Fig. 5A and B, CA (-)]. In contrast, in the presence of CA, the fluorescence intensity of YFP in the vacuole increased rapidly in a time-dependent manner [Fig. 5A and B, CA (+) + D.W.], and this increase was strongly inhibited by treatment with 3-MA [Fig. 5A and B, CA (+) + 3-MA]. These results indicate that the decrease in fluorescence intensity of YFP-NtATG8a in the cytoplasm/nucleoplasm and its increase in the vacuole in the presence of CA are suitable markers for autophagic flux in vivo in BY-2 cells.

A simple and easy method for imaging and quantification of autophagic flux using tandem fluorescent-tagged ATG8 protein. In animal cells, the site of autophagosome formation, the cytoplasm, occupies most of the cell volume. In contrast, in plants as well as yeasts, the vacuole where the autophagosomes are transported occupies substantial space of the cells. In yeast cells, the ALP (alkaline phosphatase) assay to quantify the movement of autophagic substrate from the cytoplasm to the vacuole has been established for quantitative determination of autophagic activities.\(^{31}\) However, similar method has never been reported in plants.

We showed that both reduction in the cytoplasmic fluorescence and increment in the vacuolar fluorescence of YFP-NtATG8a correlated well with the level of autophagic flux in BY-2 cells (Figs. 4 and 5). Therefore, we postulated that quantification of both the cytoplasmic and vacuolar fluorescence of YFP-NtATG8a could provide useful and convenient information for quantification of autophagic flux in living plant cells. However, simultaneous measurement of the cytoplasmic and the vacuolar fluorescence basically requires confocal fluorescence microscopy, which is laborious and time-consuming.

The pH value of the vacuolar lumen in plant cultured cells is around 5.5.\(^{32}\) The fluorescence intensity of GFP/YFP is pH dependent and GFP/YFP fluorescence often quenches when translocated into the vacuole.\(^{33}\) In contrast, the fluorescence intensity of RFP is much less affected by pH\(^{34}\) and therefore

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**Figure 1.** Visualization of sucrose starvation-induced autophagosome formation in tobacco BY-2 cells. (A) Three-day-old BY-2 cells expressing the YFP-NtATG8a construct were incubated in complete (Control) or sucrose-free medium (Starvation) for 3 h. Confocal fluorescence (a, c and e) and differential interference contrast (DIC) images (b, d) were obtained by CLSM. Arrows indicate punctate signals of YFP-NtATG8a. (A-e) Close up of the fluorescence image of YFP-NtATG8a in (A-c). Scale bars: 50 μm (a-d) and 20 μm (e). The data are representative of three experiments. (B) Time course graph of sucrose starvation-induced autophagosome formation. To quantify the levels of autophagic flux, the numbers of YFP punctate signals per 10 cells were counted at the indicated time points. The open and closed circles indicate sucrose-rich and sucrose-free conditions, respectively. Data are the means ± SE of three independent experiments. (C) Effects of PI3K inhibitors on sucrose starvation-induced autophagosome formation. Three-day-old BY-2 cells were incubated in complete or sucrose-free medium for 3 h with 3-MA (5 mM) or Wortmannin (10 μM). D.W. was used as a control. Scale bars: 50 μm. The data are representative of three experiments. (D) Quantitative levels of autophagosome formation in (C). The numbers of YFP punctate signals per 10 cells were counted. The open and closed bars indicate sucrose-rich and sucrose-free conditions, respectively. Data are the means ± SE of three independent experiments.

**Figure 2.** Subcellular localization of the GFP-NtATG8aΔG mutant protein in tobacco BY-2 cells. (A) Schematic diagrams of the C-termini of NtATG8a and its mutant defective in the Gly\(^{116}\) residue. The Gly\(^{116}\) residue of NtATG8a is shown in underlined. (B) Subcellular localization of the GFP-NtATG8aΔG mutant protein in tobacco BY-2 cells. Transgenic BY-2 cells were incubated in complete (Control) or sucrose-free medium (Starvation) for 3 h. These fluorescence images were obtained by CLSM. Scale bar: 20 μm. The data are representative of three experiments.
RFP-fusion proteins are a useful tool to monitor the translocation of proteins into the vacuole.16

Based on the difference of the fluorescence properties between YFP and RFP, we established a transgenic cell line in which ATG8 protein localized in the cytoplasm and the vacuole can be distinguished. First, we fused NtATG8a to a tandem fusion of fluorescent tags, acid-insensitive mRFP1,22,35,36 and pH-sensitive YFP, and checked the pH sensitivity of the fluorescence of these proteins by a spectrofluorometer. As expected, YFP fluorescence was drastically quenched at lower pH values, while mRFP fluorescence was not affected by pH values (Fig. S1). Nextly, we generated transgenic BY-2 cell lines (BY-HRYA8 and HRYA8ΔG) expressing HA-mRFP-YFP-NtATG8a and NtATG8aΔG fusion proteins (Fig. 6A). Under normal growth conditions, the YFP signal was detected in the cytoplasm and nucleolus of 3-d-old cultured BY-HRYA8 cells (Fig. 6B and C, Control, YFP) similarly to the BY-YA8 cells. In contrast, the RFP signal was detected in the cytoplasm, nucleolus and weakly in the vacuole (Fig. 6B and C, Control, RFP).

A recent study has shown that salicylic acid agonist BTH [benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester] induces autophagy in Arabidopsis root cells.18 When BY-HRYA8 cells were treated with 100 μM BTH, punctate signals were observed in the cytoplasm at 12 h in BY-HRYA8 cell lines (Fig. 6B, BTH), indicating that BTH induces autophagy in BY-2 cells.

When autophagy is induced by BTH treatment, the YFP fluorescence of the nucleolus decreased similarly to the BY-YA8 cells (Fig. 6C and D; YFP). In contrast, the cytoplasmic RFP fluorescence decreased but the vacuolar RFP fluorescence significantly increased (Fig. 6B and C, BTH, RFP), which is consistent with the results shown in Figure 5. In BY-HRYA8ΔG cells, all the fluorescence signals of YFP and RFP in the cytoplasm or the vacuole were not affected by BTH (Fig. 6E and F), and no accumulation of RFP in the vacuole was observed. These results strongly demonstrate that the decrease in the nucleoplasmic fluorescence of YFP/RFP and the increase in the vacuolar fluorescence of RFP can be good markers for the autophagic flux in BY-2 cells: autophagosomes fuse with the vacuole and ATG8 protein are transported from the cytoplasm to the vacuole.

These results led us to introduce the ratio of the fluorescence of RFP and YFP of the HRYA8 cells as a marker for autophagic flux. First, we quantitatively measured the fluorescence of each cell by confocal microscopy (Fig. 6G). Upon BTH treatment, the ratio of fluorescence (mRFP/YFP) dramatically increased, corresponding to the induction of autophagic flux. In contrast, (mRFP/YFP) was not affected by BTH in HRYA8ΔG cells, in which ATG8 is not translocated to the vacuole through the autophagic flux.

We further simplified the method to noninvasively monitor the fluorescence of RFP and YFP in the cell suspension using a fluorescence image analyzer (Fig. 6H) or fluorescent microscopy (Fig. S2). In HRYA8 cells, when the autophagic flux is activated by sucrose starvation, RFP fluorescence was merely affected, while YFP fluorescence dramatically decreased in a time-dependent manner.
When the autophagy is activated in YFP-NtATG8a-expressing cells, the YFP fluorescence in the cytoplasm gradually decrease for 24 h (Fig. 4), while the number of the punctate structures corresponding to the autophagosomes reached a plateau after 2–3 h and did not change afterwards (Fig. 1B). These results indicate that YFP-NtATG8a is continuously translocated from the cytoplasm to the vacuole at least for 24 h after the induction of autophagy by sucrose starvation. Therefore monitoring the decrease in cellular total fluorescence of YFP-NtATG8a could be a rough marker for autophagic flux. However, YFP-NtATG8a fluorescence can be affected by the cellular content of the protein and does not always corresponds to the activity of autophagic flux. In contrast, the present method using the two different fluorescent proteins with different pH sensitivity tandemly fused to NtATG8a enabled us to monitor the autophagic flux by normalizing the fluorescence intensity with RFP fluorescence as an internal standard.

Materials and Methods

Plant cell materials and chemicals. A tobacco BY-2 (Nicotiana tabacum L. cv Bright Yellow 2) suspension was maintained by weekly dilution (1/100) of cells in modified Linsmaier and Skoog (LS) medium, as previously reported.37 The cell suspension was agitated on a rotary shaker at 95 rpm and 25°C in the dark. Transgenic BY-2 cell lines stably expressing the YFP-NtATG8a (BY-YA8 cells), HA-GFP-NtATG8aΔG (BY-HGA8ΔG cells), HA-mRFP-YFP-NtATG8a (BY-HRYA8 cells) and HA-mRFP-YFP-NtATG8aΔG (BY-HRYA8ΔG cells) fusion proteins were maintained similarly to the non-transformed BY-2 cell line. 3-MA, wortmannin and CA were obtained from Sigma. BTH was obtained from Wako.

Plasmid construction. The YFP-NtATG8a fusion construct (pH35YG2)16 was kindly provided by Prof. Matsuoka (Kyushu University). The HA-GFP-NtATG8aΔG protein was constructed using the PCR-based cloning and Gateway recombination.
system. Briefly, a GFP-fused NtATG8a fragment was amplified by PCR from constructs containing GFP or NtATG8a with the following primers: 5'-CCG GAA TTC ATG AGC AAG GGC GAG CTG T-3' and 5'-tct ttc aag gcc ttc ccc attG CCG CCG CCC CCC TTA AGC TCG TCC ATG CCG AGA-3' for GFP containing the overlapping region of NtATG8a, 5'-gca tgg acg agc tgt caa agG CCG GCG GCG GCA TGG GGA AGG CTT TCA AAG AAG AAT-3' and 5'-CCG CTC GAG GAG CTC TCA GCT ATT TGC ACG ACC AAA GGT T-3' for NtATG8a containing the overlapping region of GFP (EcoRI and XhoI sites are underlined, and lowercase indicates the overlapping region). The PCR products were linked by PCR using the following primers: 5'-CCG GAA TTC ATG GTG AGC AAG GGC GAG-3' and 5'-CCG CTC GAG GAG CTC TCA GCT ATT TGC ACG ACC AAA GGT T-3'. The resulting product (GFP-NtATG8a) was cut with EcoRI and XhoI and subcloned into the EcoRI-XhoI site of the pGADT7 vector (Life Technologies). To generate an N-terminal HA conjugated GFP-NtATG8aΔG fragment, the HA-GFP-NtATG8a fragment was amplified by PCR using the following primers: 5'-CAC CCA TAT GAT GGC CTC CTC CGA G-3' and 5'-CAT ATG GGC GCC GGT GTA GCG G-3' for mRFP1 without the stop codon (EcoRI, XhoI and NdeI sites are underlined, and the termination codon is in bold). The PCR product was subcloned into the pENTR/SD/D-TOPO vector (Life Technologies) and then cloned into a pK7WG2 vector38 using the LR clonase reaction. The plasmids pBII121 (Life Technologies)-HA-mRFP-YFP-NtATG8a and pBII121-HA-mRFP-YFP-NtATG8aΔG were constructed by PCR-based cloning methods. YFP-fused NtATG8a and mRFP1 fragments were amplified by PCR from constructs containing the YFP-NtATG8a fragment in pH35YG2 and the mRFP1-NtATG8a fragment in pUC19, which was kindly gifted by Dr. Yoshimoto, with the following primers: 5'-CCG GAA TTC ATG GTG AGC AAG GGC GAG-3' and 5'-CCG CTC GAG GAG CTC TCA GCT ATT TGC ACG ACC AAA GGT T-3' for HA-mRFP-YFP-NtATG8a, 5'-CCG CTC GAG GAG CTC TCA AAA GGT TTT TCT ACT GCT GTA G-3' for HA-mRFP-YFP-NtATG8aΔG (BamHI and SaeI sites are underlined and the initiation and termination codons are in bold). Finally, the resulting products (HA-mRFP-YFP-NtATG8a and HA-mRFP-YFP-NtATG8aΔG) were cut with BamHI and SaeI and then cloned into the BamHI-SaeI site of the pBII121 vector (Life Technologies). The plasmid pET-21a (Merck KGaA)-ompA-His-mRFP-YFP-NtATG8aΔG was constructed by PCR-based cloning methods. To generate the signal peptide of ompA gene with a C-terminal His6 tag, two oligonucleotids: 5'-TAT GAA

Figure 5. Changes of fluorescence intensity in the vacuoles of tobacco BY-A8 cells in the presence of concanamycin a. (A) Pseudo-colored images of representative tobacco BY-2 cells expressing the YFP-NtATG8a protein at the indicated time points. Three-day-old transgenic BY-2 cells were transferred to sucrose-free medium at time 0 in the presence or absence of CA (1 μM), and with or without 3-MA (5 mM). DMSO was used as a control of 3-MA. The images were captured by CLSM and converted to a rainbow palette in the Zeiss LSM Image Browser software. Scale bar: 50 μm. The data are representative of three experiments. (B) Quantification of the fluorescence intensity in the vacuoles of tobacco BY-A8 cells. These signals were obtained quantitatively using the Image J software. Data are the means ± SE of three independent experiments.
Establishment of tobacco BY-2 cell lines stably expressing the YFP-NtATG8a, HA-GFP-NtATG8aΔG, HA-mRFP-YFP-NtATG8a and HA-mRFP-YFP-NtATG8aΔG constructs. Transformation of tobacco BY-2 cells was performed following An39 with minor modifications as follows: 4 mL of 3-d-old exponentially growing culture was transferred to 90-mm Petri dishes and incubated at 28°C with 100 µL of fresh overnight-culture of Agrobacterium tumefacens pGV2260 containing the binary vectors p7KGW2, pH35YG2 or pBII121 (Life Technologies). After a 48 h co-cultivation, the tobacco cells were washed and plated onto LS agar medium with carbenicillin (250 µg mL⁻¹) containing hygromycin (50 µg mL⁻¹) or kanamycin (50 µg mL⁻¹). Every 3–4 weeks, transformants were selected and transferred onto fresh medium for continued selection. Several cell lines were selected by examination of the YFP/GFP/RFP signals by fluorescence microscopy.

Intracellular fluorescence intensity was quantified using the confocal images. The fluorescence intensity of YFP, GFP and RFP were calculated by the average pixel value of a ROI for 200 cells per each treatment using the Image J software.

Ratiometric quantification of the fluorescence of cell suspension. Three-day-old BY-HRYA8, HRYA8ΔG and non-transformed BY-2 cells were incubated in sucrose-rich or sucrose-free medium for 20 h. The fluorescence of the cell suspension was quantified using either a fluorescence microscope or a fluoro-image analyzer as follows. At various time points, an aliquot of the cell suspensions was transferred to a 96-well plate. Cells were collected in the bottom of well by centrifugation at 100 × g for 5 min, and then both the RFP and YFP fluorescence at each well was captured with a 10 × objective using an inverted fluorescence microscope equipped with a CCD camera (BZ-9000). Alternatively, an aliquot of the concentrated cell suspension was transferred to a six-well plate, and the fluorescence of each well was detected using a 473 nm excitation laser through a 520 nm LP emission filter for YFP and a 532 nm excitation laser and 580 nm emission filter for RFP with a fluoro-image analyzer FLA3000G (GE Healthcare Bio-sciences). In both cases, the ratiometric quantification of the fluorescence was performed by dividing the average value of the YFP signals with the average value of the RFP signals using the Image J software.

Protein expression in E. coli and pH titration. pH titration assays were performed as described by Gjetting et al.35 with minor modifications as follows: The pET21a-mRFP-YFP-NtATG8aΔG plasmid was transformed into E. coli BL21AI (Life Technologies). Fresh cultures of E. coli carrying the foreign gene were cultured at 37°C until the A600 of the culture medium reached 0.5. Protein expression was induced with 1 mM IPTG and 0.2% l-arabinose, and cell growth was continued at 37°C for 16 h. The cells were harvested by centrifugation, resuspended in cold-PBS buffer with 1% tween-20, and lysed by the freeze-thaw method in accordance with the supplier’s instructions. For
pH titration of mRFP-YFP-NtATG8aΔG, the cell lysate (concentration ~20 mg mL⁻¹) was diluted to 10 volumes with 50 mM phosphate-citrate adjusted to different pH values between pH 7.0 and pH 5.4. Thereafter, the fluorescence signals derived from the cell lysate were detected with a FP6300 fluorescence spectrophotometer (JASCO). The YFP signal was excited at 514 nm and detected at 530 nm, and the mRFP signal was excited at 584 nm and detected at 610 nm.

Concluding Remarks

The system we report here applies the tandem fluorescently tagged ATG8 does not require inhibitors such as CA (or drugs) for non-invasive monitoring of autophagic flux. This is a novel way to quantitatively monitor the autophagic flow, translocation of ATG8 from the cytoplasm to the vacuole and degradation of ATG8 in the vacuole in living plant cells. Furthermore, we introduced a simple method to quantitatively monitor the autophagic flux only by measuring the fluorescence of cell suspension without microscopy. These are simple methods to monitor all the processes of the dynamics of ATG8. Not only starvation of various nutrients but also various biotic and abiotic stresses may trigger autophagy in plants. However, such physiological information is still limited and little is known on what triggers autophagy how.

The present novel method should provide a useful tool to characterize the physiological functions and molecular mechanisms of plant autophagy induced by various environmental stimuli, and screening potential chemicals to affect autophagy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/psb/article/22510/
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