TOR Is a Negative Regulator of Autophagy in *Arabidopsis thaliana*

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**Abstract**

**Background:** Autophagy is a protein degradation process by which cells recycle cytoplasmic contents under stress conditions or during senescence; a basal level of housekeeping autophagy also occurs under non-stressed conditions. Although a number of genes that function in autophagy (*ATG* genes) have been identified in plants, the upstream components that regulate the plant autophagy pathway are still obscure. Target of rapamycin (TOR) is a negative regulator of autophagy in both yeast and animals, and homologs of TOR in plants control plant growth and protein synthesis. However, a role for TOR in regulation of autophagy in plants has not been demonstrated previously.

**Methodology/Principal Findings:** In this paper we used RNA interference (RNAi) to generate transgenic plants with reduced *AtTOR* transcript level. By observing monodansylcadaverine- (MDC) and GFP-AtATG8e-labeled autophagosomes, these plants were demonstrated to have constitutive *AtATG18a*-dependent autophagy. Reverse transcriptase-PCR also showed increased expression of some *AtATG* genes in the RNAi-*AtTOR* plants. Unlike autophagy induced by starvation or salt stress, an NADPH oxidase inhibitor did not inhibit the constitutive autophagy in the RNAi-*AtTOR* lines, indicating that *AtTOR* is either downstream of or in a parallel pathway to NADPH oxidase.

**Conclusions/Significance:** Together, our results provide evidence that TOR is a negative regulator of autophagy in plants.

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**Introduction**

Upon exposure to stress conditions or during senescence, plants degrade cytoplasmic macromolecules inside the vacuole [1] by a process known as macroautophagy, or autophagy for short [2]. Autophagy is highly induced under several types of stress conditions [3,4,5,6,7,8,9]; a basal level of autophagy also exists in cells to constitutively remove unwanted materials [10,11]. Upon activation of autophagy, a double membrane structure called an autophagosome forms around the cargo to be degraded, and then delivers it to the vacuole where it is broken down and recycled [12]. Several specific functions of autophagy have been characterized in plants, including degradation of aggregated proteins in nutrient-starved plant cells [13] and proteins damaged during oxidative stress [6,14], and regulation of cell death during pathogen immune responses [15,16,17].

In *Arabidopsis thaliana*, many *AuTophaGy* (*ATG*) genes have now been characterized and found to be essential for autophagy. These have primarily been identified based on sequence similarity to yeast autophagy genes. For example, in yeast the autophagy proteins Atg2, Atg9 and Atg18 form a functional module, as Atg2 and Atg18 are required for Atg9 cycling [18]. In *Arabidopsis*, an Atg18 homolog, AtATG18a, has been shown to be required for autophagosome formation during multiple environmental stress conditions and also leaf senescence [5]. *AtATG18a* is expressed throughout the plant, with an increase in transcript level observed during conditions that upregulate the autophagy pathway [5,6]. In RNAi-*AtATG18a* transgenic plants with reduced *AtATG18a* transcript level, autophagosome formation was disrupted and the plants displayed accelerated senescence [6,16,17]. Likewise, Arabidopsis *ATG2* and *ATG9* genes also function in autophagy, suggesting that the role of these genes may be conserved between species [5,8,10].

Although the identification and characterization of *ATG* genes in plants has greatly enhanced our understanding of autophagy, the upstream regulatory components in the plant autophagy pathway are still obscure. In *Arabidopsis*, autophagy is induced by multiple abiotic and biotic stresses, including nutrient deficiency, oxidative, salt and drought stresses and pathogen invasion [5,6,16,17,19]. A potential role for NADPH oxidase in regulation of autophagy under some conditions has been suggested by inhibitor studies; NADPH oxidase inhibitors block autophagy activation during nutrient and salt stress but not during osmotic stress, indicating that NADPH oxidase-dependent and -independent regulatory pathways may exist [19]. In yeast and animals, target of rapamycin (TOR) kinase was shown to be a negative regulator of autophagy [20,21]. Tor is a phophatidylinositol 3-kinase-related kinase that functions as a Ser/Thr protein kinase [22]. It is inactivated by rapamycin, which forms a complex with FKBP12 (FK506 binding protein); this complex binds Tor and...
inhibits Tor activities [23], thus being a valuable tool in determining Tor function. Tor also controls other processes that regulate growth in response to nutrient status, for example control of translation initiation by activating the ribosomal p70 S6 kinase and inhibiting the eukaryotic translation initiation factor 4E binding protein 1 (eIF-4E BP1) [24,25,26,27].

In yeast, two TOR genes have been identified; plants, mammals and other eukaryotes have only one TOR gene [21]. In both yeast and mammals, two TOR complexes exist, TORC1 and TORC2, each of which contains distinct TOR binding partners. Only TORC1 is sensitive to rapamycin [20]. Some binding partners have been identified, including Raptor [29], which binds to substrates and presents them to Tor for phosphorylation, and LST8 [30,31], which stabilizes the TOR complex. The Atg1/Atg13 complex, which is required at an early stage of autophagy initiation to induce autophagosome formation, has been identified as a Tor substrate in metazoans. Tor functions by phosphorylating Atg13 in a nutrient-dependent manner, although the relationship between and regulation of Tor, Atg1 and Atg13 is different between yeast and metazoans [32,33,34,35].

The TOR protein is conserved in plants [36] and RAPTOR homologues have also been identified in Arabidopsis [37,38]. However, disruption of the TOR gene is lethal and causes an early block in embryo development [36], impeding the analysis of TOR function in plants. In addition, Arabidopsis is insensitive to rapamycin, and this inhibitor therefore cannot be used to study the TOR pathway in this species [36,39]. Previous research with transgenic Arabidopsis plants with increased or decreased TOR expression level [40,41] showed that growth of root and shoot was correlated with TOR expression level, indicating a role in growth regulation. A recent study in the green alga Chlamydomonas reinhardtii showed that autophagy is induced upon rapamycin treatment, suggesting that regulation of autophagy by TOR may extend to photosynthetic species[42]. However, whether TOR regulates autophagy in multicellular plants has not been investigated.

In this study, our goal was to investigate whether TOR, a negative regulator of autophagy in yeast and mammals, also plays a role in plant autophagy induction. We demonstrate that RNAi-AtTOR plants with decreased expression of AtTOR have constitutive autophagy and increased expression of some ATG genes, even in the absence of stress conditions. This constitutive autophagy is independent on the autophagy gene AtATG18a. We also show that, unlike stress-induced autophagy, an NADPH oxidase inhibitor does not inhibit the constitutive autophagy in these plants, indicating that AtTOR is most likely either downstream of or in a parallel pathway to NADPH oxidase. Taken together, our results indicate that AtTOR is a negative regulator of autophagy in Arabidopsis thaliana.

Results

Generation of RNAi-AtTOR transgenic plants

Because disruption of the AtTOR gene (At1g50030) is embryo lethal [36], to study the role of AtTOR in autophagy, RNA interference (RNAi) was used to generate plants with reduced AtTOR transcript level. An RNAi-AtTOR construct was made by linking two inverted AtTOR gene specific regions with a 1 kb GUS spacer and driven by the Cauliflower Mosaic Virus 35S promoter [43]. Arabidopsis thaliana plants were transformed with this RNAi-AtTOR construct using the floral dip method [44] and screened for kanamycin resistance. Transformants with reduced AtTOR transcript level were identified by RT-PCR using AtTOR gene specific primers (Figure 1A). Out of the 5 independent transgenic lines shown, named RNAi-1 to 5, lines RNAi-2 and RNAi-3 showed the greatest reduction in AtTOR transcript compared with the WT control, and were therefore selected for further experiments. All experiments were performed using T2 seedlings, which showed reproducible phenotypes that correlated with residual AtTOR expression level. Within a transgenic line, all individuals showed consistent AtTOR expression levels and phenotypes.

Figure 1. Generation of RNAi-AtTOR transgenic lines. A Total RNA was extracted from 7-day-old WT and five independent RNAi-AtTOR lines, RNAi-1 to RNAi-5, followed by RT-PCR analysis. 18S was used as a loading control. B The root length of 10-day-old WT, RNAi-2, RNAi-3 seedlings was measured on both MS medium and MS medium lacking nitrogen (MS-N). C The fresh weight of WT, RNAi-2, RNAi-3 seedlings grown on MS medium was measured at the stated times. *** indicates P<0.001; ** indicates P<0.01 and * indicates P<0.05. Error bars indicate standard error. doi:10.1371/journal.pone.0011883.g001
Previous research has shown that plants with a partially silenced *AtTOR* gene show reduced growth (e.g., shorter root and shoot length, smaller rosette leaves) [40,41]. To confirm that the phenotype of the RNAi lines generated were consistent with previous reports, the root length and the fresh weight of seedlings were measured. WT, RNAi-2 and RNAi-3 seeds were germinated and grown on MS medium for 10 days with the plates oriented perpendicular to the ground. The RNAi plants were found to have a small but statistically significant decrease in root length when compared with WT (Figure 1B), which is consistent with previous reports [40]. In yeast, *TOR* regulates growth and protein synthesis in response to nutrient availability [43,46]; therefore 10-day root length under nitrogen starvation conditions was also analyzed (Figure 1B). The root length of RNAi seedlings was not only decreased compared with WT, but also decreased still further under nitrogen starvation conditions compared with control nutrient-rich conditions.

To measure the fresh weight, RNAi-2, RNAi-3 and WT seedlings grown on MS media were weighed at 5, 10, 15 and 20 days (Figure 1C). From 10 days onward, both RNAi lines had a decreased fresh weight compared with the WT seedlings. To further investigate potential phenotypes of older plants in soil, RNAi-2 and WT plants grown in soil were observed throughout their lifespan. No significant differences were observed between RNAi and WT plants in seed volume, flower time and rosette size (data not shown). This differs from previous reports of reduced growth of *TOR* RNAi plants, possibly due to differences in growth conditions or extent of *TOR* silencing [40].

**RNAi-AtTOR plants have constitutive autophagy**

*TOR* is a negative regulator of autophagy in yeast and mammals [20,21]. Possible effects on autophagy in the absence of stress were therefore investigated in RNAi-*AtTOR* transgenic plants with decreased expression of *AtTOR*. Autophagy can be analyzed in *Arabidopsis* seedlings by staining with the fluorescent dye monodansylcadaverine (MDC), which selectively labels autophagosomes [47]. WT seeds and transgenic seeds of RNAi lines RNAi-1 to RNAi-5 (Figure 1A) were germinated on MS plates and grown for one week, followed by MDC staining and fluorescence microscopy to visualize autophagy (Figure 2A). In both RNAi-2 and RNAi-3 seedlings, which have the greatest reduction in *AtTOR* transcript level, constitutive autophagy was observed close to the root tip, seen as rapidly moving fluorescent puncta. Few autophagosomes were seen in transgenic seedlings with less effective reduction of transcript level (RNAi-1, RNAi-4, RNAi-5) and in WT plants. This indicates that the constitutive autophagy phenotype correlates with a greater reduction in *AtTOR* transcript level.

To confirm the constitutive autophagy in RNAi-At*TOR* plants, transgenic plants expressing the autophagosome marker GFP-AtATG8e [6] were crossed with RNAi-2 plants. Seeds from the cross were germinated on MS plates and grown for one week, then seedlings examined for autophagy activity; the autophagosomes were directly visualized under the fluorescence microscope via GFP fluorescence (Figure 2B). Consistent with the MDC staining result, autophagosomes can be observed even under control conditions in the RNAi-2 × GFP-AtATG8e plants above the root tip areas, while the GFP-AtATG8e transgenic plants have a diffuse GFP signal as expected with very few autophagosomes visible. These results further confirm the constitutive autophagy in RNAi-At*TOR* plants.

We considered two possibilities to explain the constitutive presence of autophagosomes in RNAi-At*TOR* plants: either the decreased expression of *AtTOR* leads to the constant formation of autophagosomes, or the RNAi-At*TOR* plants are deficient in delivery of autophagosomes to the vacuole for degradation. To distinguish between these two possibilities, and to further analyze the subcellular GFP-AtATG8e distribution and fate, the vacuolar H+ ATPase inhibitor concanamycin A (con A) was used to inhibit the degradation of autophagic bodies in the vacuole [9,48]. Conc A inhibits trafficking of vacuolar proteins and prevents vacuolar protein degradation [9,49]. Autophagic bodies therefore accumu-
late in the vacuole in Arabidopsis roots instead of being degraded after treatment with conc A [9]. Seven-day-old GFP-AtATG8e and RNAi-2× GFP-AtATG8e seedlings were transferred to medium containing 1 μM conc A or dimethyl sulfoxide (DMSO) as a solvent control for 12 h, and then observed by both fluorescence microscopy using a FITC filter and differential interference contrast (DIC) microscopy. In control conditions, the vacuoles in both RNAi-2× GFP-AtATG8e and GFP-AtATG8e roots rarely contain any spherical structures in DIC images. GFP-AtATG8e roots have a diffuse cytoplasmic GFP signal while RNAi-2× GFP-AtATG8e roots contain several punctate GFP-labeled structures in the cytoplasm, consistent with Figure 2B. This indicates that the DMSO solvent did not induce autophagy. After treatment with conc A, the vacuoles in the GFP-AtATG8e roots contained some weak and diffuse GFP fluorescence, and as expected, occasionally contained a few GFP puncta corresponding to spherical structures in the DIC images, as plants have a basal housekeeping level of autophagy [10,11]. In contrast, the vacuoles in the RNAi-2× GFP-AtATG8e roots contained many GFP-AtATG8e-labeled puncta and spherical structures in the DIC images which have been shown previously to be autophagic bodies [9]. By comparing the GFP fluorescence with the DIC images in RNAi-2× GFP-AtATG8e roots after conc A treatment, most of the GFP-AtATG8e signal and the spherical structures localize inside the vacuole. This suggests that after treatment with conc A, autophagic bodies accumulate in the vacuole in the RNAi-2× GFP-AtATG8e roots instead of being degraded. The accumulation of autophagic bodies in the vacuoles of RNAi-2× GFP-AtATG8e plants suggests that autophagosomes are formed and successfully transferred into vacuoles in these plants. These results indicate that the constitutive autophagy in RNAi-AtTOR seedlings is most likely caused by the increased formation of autophagosomes, rather than a deficiency in delivery to or fusion between autophagosomes and vacuoles. These data therefore suggest that AtTOR may negatively regulate autophagy in Arabidopsis.

**Some ATG genes are up-regulated in the RNAi-AtTOR plants**

A number of genes have been shown to be required for autophagy in Arabidopsis and several are upregulated under conditions that induce autophagy [50]. Previously, we have shown that under starvation, oxidative, salt and osmotic stresses, autophagy induction is correlated with the upregulation of the AtATG18a gene [At3g62770] [5,6]. In the RNAi-AtTOR plants, autophagy was observed even under control conditions. Therefore the expression of the AtATG18a gene was analyzed in these plants. AtATG9 [At2g31260] and the AtATG8 gene family are also essential for autophagy in Arabidopsis [8,9]; the expression of AtATG9 and several AtATG8 [AtATG8b [At4g04620], AtATG8e [At2g45170], AtATG8f [At4g16520], AtATG8g [At3g06420]] genes was also analyzed. WT and RNAi-2 seedlings were germinated on control MS plates and grown for one week, RNA was extracted from 8 mm of the root tips from seven-day-old WT and RNAi-2 lines, followed by RT-PCR analysis of the indicated genes. 18S RNA was used as a control for equal RNA levels in each sample. The expression level of each gene was quantified by densitometry of bands from at least three independent replicates, with AtATG18apro: GUS 0 h value set as 1.

** indicates P<0.01 and * indicates P<0.05. Error bars indicate standard error.

**Figure 3. Some ATG genes are up-regulated in RNAi-AtTOR plants.** A RNA was extracted from 8 mm of the root tips from seven-day-old WT and RNAi-2 lines, followed by RT-PCR analysis of the indicated genes. 18S was used as a loading control. B Densitometry was used to quantify the relative amounts of RT-PCR product from at least three independent replicates, with the wild-type control value set as 1. ** indicates P<0.01 and * indicates P<0.05. Error bars indicate standard error. C 7-day-old AtATG18aprol: GUS and RNAi-2× AtATG18aprol:GUS seedlings were collected and submerged in GUS staining solution for 16 h, destained in 70% ethanol for 16 h and observed with a light microscope. Scale bar = 1 mm. D 7-day-old AtATG18aprol: GUS and RNAi-2× AtATG18aprol:GUS root tissue extracts were added to 1 mM MUG solution to assay GUS activity. At 0 h, 0.5 h, 1 h, 2 h, 4 h, 6 h and 16 h, the reactions were terminated with 0.2 M Na2CO3 followed by measurement of MU fluorescence. Data was collected from 3
independent RT-PCR experiments, with the expression in WT plants set to 1. The mRNA level of both AtATG18a and AtATG9 was significantly higher in the RNAi-2 plants than in WT, but no difference was seen in expression of any of the AtATG6 genes tested compared with WT plants (Figure 3B). The same RT-PCR experiment was also performed with the RNAi-3 line, with identical results to the RNAi-2 line (data not shown).

Previously, AtATG18apro:GUS transgenic plants were generated, in which the GUS reporter gene was expressed under the control of the AtATG18a promoter as an alternative way to observe the expression of AtATG18a [19]. In these plants, GUS activity is very low under control conditions, but increases substantially upon induction of autophagy. To confirm the RT-PCR result, AtATG18apro:GUS plants were crossed with RNAi-2 plants. The seeds were germinated on MS plates and grown for one week; the seedlings were then collected and submerged in GUS staining solution for 16 h, destained in 70% (v/v) ethanol for 16 h and observed with a light microscope (Figure 3C). In the AtATG18apro:GUS plants, the roots have no visible GUS activity under control conditions. In contrast, in the RNAi-2×AtATG18apro:GUS plants, GUS activity is evident throughout the roots. This indicates that the AtATG18a gene is up regulated in the RNAi-2×AtATG18apro:GUS plants.

To further quantify the GUS staining results in Figure 3C, GUS activity was measured using the fluorometric substrate 4-methylumbelliferyl-β-D-glucuronide (MUG) [51]. MUG can be hydrolyzed by GUS to produce the fluorochrome 4-methylumbellifereone (MU). AtATG18apro:GUS and RNAi-2×AtATG18a-pro:GUS seeds were germinated and grown on MS medium for 7 days with the plates oriented perpendicular to the ground. 0.05 g of root tissue was excised, followed by measurement of GUS activity by monitoring the fluorescence of MU at 0 h, 0.5 h, 1 h, 2 h, 4 h, 6 h, and 16 h (Figure 3D). In both AtATG18apro:GUS and RNAi-2×AtATG18apro:GUS plants, the fluorescence increased gradually over time. However, the fluorescence increase was much greater in the RNAi-2×AtATG18apro:GUS plants than in RNAi-2×AtATG18apro:GUS plants. After 6 h, the fluorescence in RNAi-2×AtATG18apro:GUS plants was more than 3 fold higher than in the AtATG18apro:GUS plants, and increased to almost 8 fold higher after 16 h. This again shows that the RNAi-2×AtATG18apro:GUS plants have higher GUS activity than the AtATG18apro:GUS plants, which is consistent with the GUS staining results.

These results together indicate that the AtATG9 and AtATG18a genes are up-regulated in the RNAi-AtTOR plants in the absence of stress.

**AtTOR-regulated autophagy is dependent on AtATG18a**

Since AtATG18a is required for autophagosome formation during stress and senescence, RNAi-AtATG18a plants with reduced AtATG18a transcript level are defective in autophagosome formation [5]. To determine whether AtTOR-regulated autophagy requires AtATG18a, the RNAi-2 plants were crossed with RNAi-AtATG18a plants. The seeds were germinated on MS plates and grown for one week, followed by MDC staining. As shown in Figure 4A, RNAi-2 plants have constitutive autophagy in control conditions, while RNAi-2×RNAi-AtATG18a plants have no observable autophagy. This indicates that autophagy induced upon inactivation of AtTOR requires AtATG18a, in common with stress-induced autophagy.

To further confirm the absence of autophagosome formation in the RNAi-2×RNAi-AtATG18a plants, conc A was used to assess the accumulation of autophagic bodies in the vacuole. Seven-day-old RNAi-2 and RNAi-2×RNAi-AtATG18a seedlings were transferred to medium containing 1 mM conc A or DMSO as a control for 12 h, and then observed by DIC microscopy (Figure 4B). In control conditions, spherical structures were absent in both RNAi-2 and RNAi-2×RNAi-AtATG18a vacuoles. After treated with conc A, spherical structures accumulated in the RNAi-2 vacuoles but not the RNAi-2×RNAi-AtATG18a vacuoles. These data again suggest the constitutive formation of autophagosomes in the RNAi-2 but not the RNAi-2×RNAi-AtATG18a plants. This confirms that AtTOR-regulated autophagy is dependent on AtATG18a.

**NADPH oxidase inhibitor does not inhibit autophagy in RNAi-AtTOR plants**

Previous results showed that autophagy is induced by nutrient deprivation, senescence, high salinity, oxidative and osmotic stresses [3,6,19]. All of these conditions elevate the cellular ROS (reactive oxygen species) levels, which can act as signal molecules to activate stress response and defense pathways [52,53]. Plasma membrane NADPH-dependent oxidase is a major source of signaling ROS. The NADPH oxidase inhibitors DPI (diphenylene iodonium) and imidazole [54] inhibit nutrient and salt stress-induced autophagy, whereas autophagy induced by osmotic stress is insensitive to these inhibitors, indicating that autophagy is regulated by NADPH oxidase-dependent or –independent pathways, determined by the induction conditions [19].

To determine whether AtTOR works downstream or upstream of NADPH oxidase in the autophagy signaling pathway, the effect of the NADPH oxidase inhibitor imidazole [54] on RNAi-AtTOR plants was tested. One week old RNAi-2 and WT seedlings, germinated on control MS plates, were transferred to liquid MS medium plus or minus 20 mM imidazole. WT seedlings transferred to liquid MS medium plus 0.16 M NaCl plus or minus 20 mM imidazole were used as positive controls. To observe autophagosomes, MDC staining was performed (Figure 5A). For the WT seedlings, no autophagosomes were observed either with or without imidazole treatment under the...
control conditions, demonstrating that as expected, imidazole does not induce autophagy. Upon exposure to 0.16 M NaCl, numerous autophagosomes were seen in WT plants in the absence of imidazole, whereas in the presence of imidazole, no autophagosomes were present, confirming that imidazole inhibits autophagy induction under salt stress conditions, consistent with previous results [19]. For the RNAi-2 plants, imidazole had no effect on the constitutive autophagy seen in these lines, with numerous autophagosomes seen both in the presence and the absence of this inhibitor. This indicates that the NADPH oxidase inhibitor imidazole does not inhibit the observed autophagy in the RNAi-2 plants.

To confirm the MDC staining results (Figure 5A), one week old RNAi-2 × GFP-AtATG8e and control GFP-AtATG8e seedlings were transferred to liquid MS medium plus or minus 20 mM imidazole. GFP-AtATG8e seedlings transferred to liquid MS medium plus 0.16 M NaCl plus or minus 20 mM imidazole were used as positive controls. Autophagosomes can be directly visualized via GFP fluorescence (Figure 5B). In the control condition, no autophagosomes were observed either with or without imidazole treatment. Autophagy was induced in the presence of 0.16 M NaCl, whereas adding imidazole inhibited the autophagy induction. In RNAi-2 × GFP-AtATG8e seedlings, GFP-labeled autophagosomes were seen both with and without the imidazole. These results are consistent with the MDC staining, again suggesting that imidazole does not inhibit autophagy in the RNAi-2 plants.

To confirm that the GFP-AtATG8e structures in the RNAi-2 plants are autophagosomes, one week old RNAi-2 × GFP-AtATG8e and control GFP-AtATG8e seedlings were incubated in the presence of 1 μM conc A or DMSO for 12 h, each plus or minus 20 mM imidazole. Autophagic bodies were then observed by both fluorescence microscopy using a FITC filter and differential interference contrast (DIC) microscopy (Figure 5C). In both the presence and absence of imidazole, many punctate GFP-AtATG8e signals were observed. After adding conc A, in both the presence and absence of imidazole, many punctate GFP-labeled spherical structures were observed. By comparing the GFP fluorescence with DIC images, both GFP-AtATG8e and the spherical structures in DIC images were localized inside the vacuoles. This indicates that conc A treatment led to the accumulation of autophagic bodies in the vacuoles both with and without imidazole treatment, again confirming that the NADPH oxidase inhibitor imidazole does not inhibit the constitutive generation of autophagosomes in the RNAi-2 × GFP-AtATG8e plants. Together, these results suggest that AtTOR is either downstream of NADPH oxidase, or works in an NADPH oxidase-independent pathway.

Discussion

Although several autophagy-related genes have been identified and characterized in plants during the past decade, the upstream regulatory components in the autophagy pathway are still unclear.
Autophagy has been reported to be activated in the unicellular green alga *Chlamydomonas reinhardtii* upon rapamycin treatment [42], suggesting that TOR negatively regulates autophagy in this species. However, due to the insensitivity of *Arabidopsis* to rapamycin and the lethality of the knockout mutant, the study of TOR function in the autophagy pathway in land plants has been difficult [36,39]. To overcome these obstacles, we generated RNAi-AtTOR transgenic plants to reduce AtTOR transcript level. These lines were first confirmed to have reduced growth phenotypes (Figure 1B), consistent with previous research [40], and the reduced root growth phenotype was enhanced upon nitrogen starvation. TOR regulates multiple pathways to coordinate the response to nutrient availability, and decreased TOR activity during starvation is predicted to both increase the activity of the autophagy pathway and decrease growth-promoting pathways such as translation [55]. In two independent RNAi-AtTOR lines, constitutive autophagy was observed in roots under control conditions, in contrast to WT plants in which autophagosomes are rarely seen in the absence of stress (Figure 2). The autophagy-related genes AtATG18α and AtATG9 were shown to have an increased transcript level in the RNAi-AtTOR lines (Figure 3A, 3B). AtTOR-regulated autophagy was dependent on AtATG18α, as RNAi-AtTOR×RNAi-AtATG18α plants do not show constitutive autophagy (Figure 4). Finally, an NADPH oxidase inhibitor did not inhibit autophagy in RNAi-AtTOR plants as it does under nutrient and salt stress conditions (Figure 5), suggesting that AtTOR is either downstream of or in a pathway that is parallel to NADPH oxidase.

There is considerable evidence that TOR is an upstream regulator of autophagy in numerous species. Tor suppresses starvation-induced autophagy in the *Drosophila* fat body [56] and yeast Tor controls autophagy by responding to nutrient availability [32]. In the green alga *Chlamydomonas reinhardtii*, which is sensitive to rapamycin, inhibition of TOR by rapamycin leads to an increase in vacuole size and formation of ATG8-decorated autophagosomes, consistent with a role in regulation of autophagy [42,57]. In plants, TOR was shown previously to sense nutrient availability and regulate plant growth [40]. However, little evidence has directly shown a relationship between TOR and autophagy in multicellular plants. In this study, we provide evidence that TOR also controls autophagy, in addition to growth, in plants.

When autophagy is induced by stress conditions, autophagosomes are observed in the roots [5,6]. Autophagosomes were observed in the area close to the root tip in the RNAi-AtTOR seedlings, primarily in the division and elongation zones. TOR is most highly expressed in rapidly dividing and growing tissues such as meristems, embryos and endosperm [36]. It is therefore likely that the decreased expression of the AtTOR gene has the greatest effect on rapidly growing and expanding cells and therefore caused the induction of autophagy in these cells. It is clear from this and previous research [36,40] that TOR signaling is only partially suppressed in the RNAi lines described, and a complete disruption of TOR activity may be necessary to see the full effect of TOR inhibition on autophagy. In addition, the fluorescence assays used are technically difficult in shoot tissues due to the higher autofluorescence, making assessment of possible induction of autophagy in shoot cells problematic. It is possible that autophagy is also activated in rapidly growing shoot tissues in the RNAi lines. The GUS staining results shown in Figure 3C suggest an increased expression of AtATG18α throughout most of the root in the RNAi-AtTOR plants; this suggests that AtTOR activity is affected in upper parts of the root as well as root tips. One explanation is that the increased expression of AtATG18α in certain root regions does not necessarily correspond to the formation of autophagosomes. Further reduction of AtTOR expression level may be required for autophagosome formation, or other factors may also contribute to regulation of autophagy induction.

In general, Tor proteins function as Ser/Thr protein kinases [22]. Tor signals through a combination of direct phosphorylation of downstream targets and repression of phosphatase activity [58]. Some possible substrates have already been identified in plants, such as a meiosis signaling molecule AML1 (*Arabidopsis* Mei2-like 1) [59] and EBPI (*EzbB-3* epidermal growth factor receptor binding protein) [60] in *Arabidopsis*, and a translation regulator S6K (ribosomal p70 S6 kinase) [39,61], but their relationship to autophagy is not known. The Atg1/Atg13 complex has been shown to be a Tor substrate in yeast and metazoans. Tor functions by phosphorylating Atg13 in a nutrient-dependent manner, although the regulation and functions of Tor, Atg1 and Atg13 are divergent when comparing different species, complicating the generation of an overall model for TOR function [32,33,34,35]. In *Arabidopsis*, three putative Atg1 homologues and two putative Atg13 homologues have been identified [62], but their functions and interactions with TOR are still unknown. Our study shows that at least some AtATG genes are under the control of AtTOR in the autophagy pathway. However, to better understand and further investigate the components in the autophagy pathway, future experiments may focus on testing autophagy induction by inactivating or activating AtTOR and identifying its downstream targets.

### Materials and Methods

#### Plant materials and growth conditions

*Arabidopsis* seeds were surface sterilized with 0.1% (v/v) Triton X-100 and 33% (v/v) bleach solution for 20 min, followed by cold treatment for at least 2 days. Seedlings were grown at 22°C under long day conditions (16 h light) on nutrient solid MS medium [Murashige–Skoog Vitamin and Salt mixture (Caisson, North Logan, UT, USA), 1% (w/v) sucrose, 2.4 mM MES (pH 5.7) and 0.8% (w/v) phytagar].

#### Generation of RNAi-AtTOR transgenic plants

The RNAi-AtTOR construct was generated as described by Chuang and Meyerowitz [43]. Sense and antisense fragments of *AtTOR* were amplified by RT-PCR (reverse transcription-polymerase chain reaction) using gene-specific primers (Table 1). The sense fragment, a 1-kb GUS spacer gene fragment, and the antisense fragment were ligated into the plant T-DNA binary vector pCGN and driven by the Cauliflower Mosaic Virus 35S promoter. The RNAi construct was introduced into *Agrobacterium tumefaciens* strain GV2260 by electroporation [63], and then into *Arabidopsis thaliana* Columbia-0 plants by Agrobacterium-mediated transformation using the floral dip method [44]. The expression level of the AtTOR gene in each transformant was determined by RT-PCR and homozygous T2 transformant seeds with reduced AtTOR mRNA level were used for further studies.

RNAi-2×RNAi-AtATG18α plants were generated by crossing RNAi-2 plants with RNAi-AtATG18α plants [5]. RNAi-2×AtATG18α promoter:GUS plants were generated by crossing RNAi-2 plants with AtATG18α promoter:GUS plants [19].

#### RT-PCR analysis of AtTOR, AtATG18α and AtATG9

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and followed by DNase I treatment. The final RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer; 1 µg RNA was used to generate cDNAs.
using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). An oligo dT primer was used for AtATG18a and the AtATG8s (b, e, f, h); gene specific primers were used for AtTOR and AtATG9 (Table 1). Gene specific primers used for PCR are also shown in Table 1. The PCRs were run for 28 cycles with annealing temperatures of 50, 55, 55 and 60°C and extension times of 1, 1, 1 and 4 min for AtTOR, AtATG18a, AtATG8s and AtATG9, respectively.

RT-PCR signals were quantified by densitometry. Individual bands were analyzed using Quantity One software (Bio-Rad Laboratories; Hercules, CA) using the volume analysis function. The relative signals were calculated with the wild-type control value set as 1 for each gene individually. The results shown are an average of at least 3 independent experiments.

MDC staining and microscopy

Wild-type and RNAi-AtTOR seedlings were stained with MDC as previously described [47]. Seedlings were incubated with 0.05 mM MDC for 10 mins, washed 3 times with phosphate buffered saline (PBS) and observed using a Zeiss Axioplan II microscope. MDC fluorescence was visualized using a DAPI-specific filter and GFP fluorescence was visualized using a FITC-specific filter.

Concanamycin A Treatment

Seven-day-old seedlings grown on MS plates were transferred to MS liquid medium containing 1 µM concanamycin A or dimethyl sulfoxide (DMSO) as a solvent control for 12 to 16 h in the dark. The roots were mounted in water and then observed by fluorescence and differential interference contrast (DIC) microscopy.

GUS staining

Seedlings were collected and submerged for 16 h in the following staining solution: Triton/ethanol stock (Triton X-100: ethanol: water; 1:4:5), 0.5 M KPO₄ buffer (pH 7.0), 0.1 M ferricyanide solution (pH 7.0), 0.1 M ferrocyanide solution (pH 7.0), 10 mg/ml bromo-4-chloro-3-indolyl-b-D-glucopyranoside in dimethyl sulfoxide (5:470:2:225). Plants were washed once with 70% (v/v) ethanol, destained in 70% ethanol for 16 h and observed by light microscopy [64].

Fluorometric assay

Fluorometric reactions for analysis of GUS activity were performed according to Jefferson et al. [51]. 0.05 g of root tissue was homogenized in 50 µl extraction buffer (0.1 M Tris-HCl pH 7.5, 0.3 M sucrose, 1 mM EDTA, 0.1 mM PMSF) with liquid nitrogen. Plant extracts were centrifuged for 5 min at 4°C at 14,000 rpm. The supernatants were added to 1 mM MUG solution and at regular time intervals the reactions were terminated with 0.2 M Na₂CO₃ solution. Fluorescence was then measured with a BIO-TEK Synergy HT multi-detection microplate reader, with excitation at 360 nm and emission at 460 nm. The microplate reader was calibrated with freshly made 4-methyl umbelliflorine (MU) standards of 0.5 nm, 5 nm, 25 nm and 50 nm.

Inhibitor treatment

7-day-old seedlings grown on nutrient solid MS medium were transferred to MS liquid medium plus or minus 20 mM imidazole for 4 h.

Acknowledgments

We thank Dr. Harry (Jack) T. Horner, Randall Den Adel and Tracey M. Pepper for assistance with the microscopy, Xi Chen and Ting Li for assistance with the MUG assay, and Anthony L. Contento for discussions and comments on the manuscript.

Author Contributions

Conceived and designed the experiments: YL DB. Performed the experiments: YL DB. Analyzed the data: YL DB. Wrote the paper: YL DB.

Table 1. Primers used for generating the RNAi-AtTOR construct and for RT-PCR analysis of AtTOR, AtATG18a, AtATG9 and AtATG8s.

| Primer | Forward | Reverse |
|--------|---------|---------|
| Sense  | EcoRI5'-AGCGGATATCATGTTCTCTGCTTCCAATC-3' | XbaI5'-TCTAGCTCTAGACCAATTCGCTCAATCT-3' |
| Anti-sense | EcoRI5'-AGCGAGTATCATGTCTTCCAATC-3' | BamHI5'-AGCGGATATCATGTTCTCTAGATC-3' |
| AtTOR  | 5'-TCGGTCGTCACCTCCATATTCCGACTCTCATG-3' | 5'-TATCTAGCTCTAGGAAATCCGACTCTCATG-3' |
| AtATG18a | 5'-TCGGTCGTCACCTCCATATTCCGACTCTCATG-3' | 5'-TATCTAGCTCTAGGAAATCCGACTCTCATG-3' |
| AtATG9  | 5'-TCGGTCGTCACCTCCATATTCCGACTCTCATG-3' | 5'-TATCTAGCTCTAGGAAATCCGACTCTCATG-3' |
| AtATG8b | 5'-TCGGTCGTCACCTCCATATTCCGACTCTCATG-3' | 5'-TATCTAGCTCTAGGAAATCCGACTCTCATG-3' |

References

1. Basham DC (2007) Plant autophagy—more than a starvation response. Curr Opin Plant Biol 10: 587–593.
2. Klonosky DJ (2005) Autophagy. Curr Biol 15: R282–283.
3. Thompson AR, Doelling JH, Suttangkakul A, Vierstra RD (2005) Autophagic nutrient recycling in Arabidopsis directed by the ATG8 and ATG12 conjugation pathways. Plant Physiol 138: 2097–2110.
4. Doelling JH, Walker JM, Friedman EM, Thompson AR, Vierstra RD (2002) The APG8/12-activating enzyme APG7 is required for proper nutrient recycling and senescence in Arabidopsis thaliana. J Biol Chem 277: 33105–33114.
5. Xiong Y, Contento AL, Basham DC (2005) AtATG18a is required for the formation of autophagosomes during nutrient stress and senescence in Arabidopsis thaliana. Plant J 42: 535–546.
6. Xiong Y, Contento AL, Nguyen PQ, Basham DC (2007) Degradation of oxidized proteins by autophagy during oxidative stress in Arabidopsis. Plant Physiol 143: 291–299.
7. Moriyasu Y, Ohsumi Y (1996) Autophagy in tobacco suspension-cultured cells in expression among the starch debranching enzyme genes AtISA1, AtISA2, and AtISA8. Mol Biol Cell 14: 1204–1220.

8. Kamada Y, Funakoshi T, Shintani T, Nagano K, Ohsumi M, et al. (2000) Tor kinases are in distinct membrane-associated protein complexes in Arabidopsis macroautophagy. Mol Biol Cell 11: 567–577.

9. Anderson GH, Veit B, Hansen MR (2005) The Arabidopsis AtRaptor genes are essential for post-embryonic plant growth. BMC Biol 3: 12.

10. Deprosi D, Troang LN, Hagiwara A, Chang CT (2005) Arabidopsis homolog of RAPTOR/KOG1 is essential for early embryo development. Biochem Biophys Res Commun 329: 444–450.

11. Mahfouz MM, Kim S, DeLaurier AJ, Verma DP (2006) Arabidopsis TARGET OF RAPAMYCIN interacts with RAPTOR, which regulates the activity of S6 kinase in response to osmotic stress signals. Plant Cell 18: 477–490.

12. Deprosi D, Troang LN, Hagiwara A, Chang CT (2007) The Arabidopsis TOR kinase links plant growth, yield, stress resistance and mRNA translation. EMBO Rep 8: 864–870.

13. Sornami R, Yao L, Menand B, Ennar M, Lecampion C, et al. (2007) Saccharomyces cerevisiae FKBP12 binds Arabidopsis thaliana TOR and its expression in plants leads to rapamycin susceptibility. BMC Plant Biol 7: 26.

14. Perez-Perez ME, Floresco F, Crespo JL (2010) Inhibition of target of rapamycin signaling and stress autocrine activity in pathogen-associated cell death response. Autophagy 4: 20–27.

15. Shlomai J, Lohr S, Park J, Wang C, Wang J, et al. (2009) Arabidopsis homologs of yeast autophagy genes, homologs of plant autophagy in Arabidopsis root tip cells. Plant Cell Physiol 47: 1641–1652.

16. Xiong Y, Contejo JL, Basham DC (2007) Duration of autophagy results in constitutive oxidative stress in Arabidopsis. Autophagy 3: 257–258.

17. Raggirola F, Klonios DJ (2005) Autophagosomes: biosynthesis from scratch? Curr Opin Cell Biol 17: 415–422.

18. Toyooka K, Moriyasu Y, Takeuchi M, Fukuda H, et al. (2006) Protein aggregates are transported to vacuoles by a macroautophagic mechanism in nutrient-starved plant cells. Autophagy 2: 96–106.

19. Patel S, Dinesh-Kumar S (2008) Arabidopsis ATG6 is required to limit the pathogen-associated cell death response. Autophagy 4: 20–27.

20. Liu Y, Scliff M, Cazmneke K, Talbycz Y, Levine B, et al. (2005) Autophagy regulates programmed cell death during the plant immune response. Cell 121: 567–577.

21. Hofsin D, Schultz-Larsen T, Joensen J, Tsitsigiannis D, Petersen NH, et al. (2002) Autophagic components contribute to hypersensitive cell death in Arabidopsis. Cell 110: 773–783.

22. Pattingre S, Expert L, Biard-Piechaczyk M, Cogdogo P (2008) Regulation of macroautophagy by mTOR and Beclin 1 complexes. Biochemie 90: 513–523.

23. Hochstrasser M (1997) Is a lipid kinase not a lipid kinase? When it is a protein kinase. Cell 83: 1–4.

24. Hall MN (2008) TOR—what does it do? Transplant Proc 40: S5–8.

25. Thomas G, Hall MN (1997) TOR signalling and control of cell growth. Curr Opin Cell Biol 9: 769–777.

26. Hara K, Yonezawa K, Weng QF, Kozoloski MT, Belham C, et al. (1998) Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BPI through a common effector mechanism. J Biol Chem 273: 14904–14909.

27. Casarins GP, Schmerel T, Haghjat A, Hellewell SB, Hall MN, et al. (2000) Eap1p, a novel eukaryotic translation initiation factor 4E-associated protein in Saccharomyces cerevisiae. Mol Cell Biol 20: 4604–4613.

28. Urban J, Soulard A, Huber A, Lappman S, Mukhopadhyay D, et al. (2007) Sch9 is a major target of TORC1 in Saccharomyces cerevisiae. Mol Cell 26: 663–674.

29. Loewith R, Jacinto E, Wullschleger S, Lorberg A, Crespo JL, et al. (2002) Two TOR kinases in Arabidopsis. Mol Biol Cell 13: 773–783.

30. Hara K, Maruki Y, Long X, Yoshino K, Oshiro N, et al. (2002) Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell 110: 477–487.

31. Wedaman KP, Reinke A, Anderson S, Yates J, 3rd, McCaffery JM, et al. (2003) Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 binding partner of target of rapamycin (TOR), mediates TOR action. Cell 110: 177–189.

32. Kim DH, Sarbassov DD, Ali SM, Latexk RR, Guntur KV, et al. (2003) GbetaL, a sensitive interaction between raptor and mTOR. Mol Cell 11: 895–904.

33. Chang YY, Neufeld TP (2009) An Atg1/Atg13 complex with multiple roles in neuronal development. J Biol Chem 284: 13792–13799.

34. Hosokawa N, Hara T, Kaizuka T, Kishi C, Takamura A, et al. (2009) Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. Mol Biol Cell 20: 1981–1991.

35. Inoue Y, Suzuki T, Takeuchi M, Fukuda H, et al. (2006) AtATG genes, homologs of yeast autophagy genes, are involved in constitutive autophagy in Arabidopsis root tip cells. Plant Cell Physiol 47: 1641–1652.

36. Menad B, Destou N, Nussaume L, Berger F, Bouchaud D, et al. (2002) Expression and disruption of the Arabidopsis TOR [target of rapamycin] gene. Proc Natl Acad Sci U S A 99: 6422–6427.

37. Deproso D, Troang LN, Hagiwara A, Chang CT (2005) Arabidopsis AtRaptor genes are essential for post-embryonic plant growth. BMC Biol 3: 12.