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Review

Function and regulation of macroautophagy in plants

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ABSTRACT

The plant vacuole is a major site for the degradation of macromolecules, which are transferred from the cytoplasm by autophagy via double-membrane vesicles termed autophagosomes. Autophagy functions at a basal level under normal growth conditions and is induced during senescence and upon exposure to stress conditions to recycle nutrients or degrade damaged proteins and organelles. Autophagy is also required for the regulation of programmed cell death as a response to pathogen infection and possibly during certain developmental processes. Little is known about how autophagy is regulated under these different conditions in plants, but recent evidence suggests that plants contain a functional TOR pathway which may control autophagy induction in conjunction with hormonal and/or environmental signals.

1. Introduction

In plant cells, the vacuole is a major site for the bulk degradation of macromolecules, both under normal growth conditions and during exposure to abiotic and biotic stresses [1]. Macroautophagy, often termed simply autophagy, appears to be the predominant pathway for the transfer of organelles, organelle fragments and cytosolic macromolecules into the vacuole for degradation by the numerous proteases, lipases, nucleases and other hydrolytic enzymes present inside this compartment [2,3]. A basal level of autophagy functions constitutively as a housekeeping process for the breakdown of damaged or unwanted cellular components [4,5], whereas it is induced to a high level during certain stresses. Much of the research on plant autophagy has focused on its role as a response to nutrient stress, in which the pathway acts to break down and recycle macromolecules to counteract starvation [6,7].

The pathway for the transport of material to the vacuole by autophagy has been well defined morphologically in many species, including several plants [6–10] (Fig. 1). Cup-shaped membranes are initially seen associated with the material destined for degradation (Fig. 1, stage 1). These membranes expand (2) and fuse to completely surround the material, generating a double membrane vesicle known as an autophagosome (3). The autophagosome then moves to the vacuole, upon which the outer membrane fuses with the tonoplast (4). The remaining single membrane structure, an autophagic body, is transferred into the vacuole (5) and degraded (6). The breakdown products are presumably exported from the vacuole to the cytoplasm for re-use (7), although little is known about this step in plants.

A number of genes required for autophagy have been identified, initially through genetic screens for autophagy-defective mutants in yeast [11–13]. Characterization of the function of the encoded proteins has provided insight into the mechanism of autophagosome formation, which appears to be conserved throughout eukaryotes, including plants [14–17]. Major components conserved in plant cells include two ubiquitin-related protein conjugation systems [16,18–21], a membrane protein and its recycling system required for initiation of autophagosome formation [5,17, 22] and a phosphatidylinositol 3-kinase (PI3-kinase) complex [23]. Much of the research in plants on these components has focused on the model plant *Arabidopsis thaliana*, including the isolation of knockout mutants with disruptions in homologs of yeast autophagy genes [5,16–20,22,24–26], although autophagy-related genes have now also been identified in some crop species [27–29]. These mutants show phenotypes expected of nutrient recycling defects, being hypersensitive to macronutrient deprivation and displaying premature leaf senescence [16–19,22,25,26,30], suggesting that autophagy is an important component of the recycling pathway.

Several recent reviews have described the identification and function of genes involved in autophagy in plants [1,15,31,32]. Here, I will focus on the activation of autophagy in response to different stimuli and the potential regulatory pathways that control this activation.

2. Hormonal and environmental regulation of autophagy

Autophagy has been known for some time to function in response to nutrient starvation in plants [6,7]. Recent reports suggest that autophagy and autophagy components are also involved in the response to other types of environmental stress conditions.

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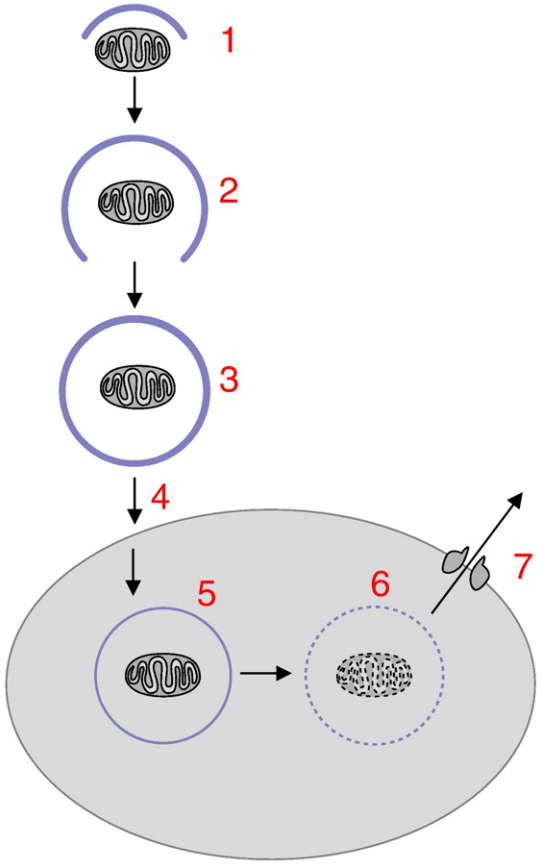

Fig. 1. Pathway for degradation of cytoplasmic material by autophagy in plant cells. Portions of cytoplasm, for example a mitochondrion as shown here, are enwrapped by a double membrane and delivered to the vacuole for recycling. See text for details of each numbered step.

Autophagy is strongly induced in response to oxidative stress (Fig. 2), and *Arabidopsis* plants defective in autophagy are hypersensitive to reactive oxygen-producing agents [33]. This is at least partly due to the accumulation of oxidized proteins, which in the absence of autophagy accumulate to high levels as they cannot be efficiently degraded. Autophagy has also been shown to deliver protein aggregates to the vacuole for degradation [34], which may be important as a general response to many different stress conditions.

Studies of a family of genes involved in autophagy in *Arabidopsis* have provided clues to other possible conditions that activate autophagy. ATG8 is a critical component of the autophagy pathway and is lipid-modified upon activation of autophagy, leading to its association with autophagosomes [21,28,35,36]. GFP-ATG8 fusion proteins have therefore been used as markers for autophagy in plants [20,25,26,37] as they are in other species [38]. *Arabidopsis* contains nine genes encoding ATG8-like proteins, and evidence suggests that all of them may function in the autophagy pathway [21,26]. The regulation of ATG8 family members has been examined by RT-PCR [25], by analysis of microarray data [25,39] and using transgenic plants expressing promoter-GUS fusions to five of the ATG8 proteins [20]. While the genes are generally broadly expressed, some differences in their pattern of expression and responses to sucrose starvation were seen, suggesting that the genes may be regulated independently and function under different conditions.

One of these ATG8 proteins, ATG8f, was expressed as a fusion with GFP and a C-terminal HA-tag in transgenic *Arabidopsis* plants under the control of the strong, semi-constitutive 35S promoter from Cauliflower Mosaic Virus [40]. The transgenic plants were slightly larger than control plants and showed accelerated flowering under nutrient limiting conditions, raising the possibility of enhanced autophagy. The plants were also more sensitive to both salt and osmotic stresses. In wild-type plants, high salt concentrations lead to cell death in the primary meristem, causing a reduced growth rate which in turn contributes to greater salt tolerance [41]. The authors suggest that enhanced autophagy in the transgenic lines may prevent this cell death, and therefore compromise salt tolerance [40]. The lines also had altered responses to the plant hormone cytokinin, which regulates nitrogen metabolism, movement and root-shoot communication [42], raising the possibility of hormonal regulation of autophagy in response to nitrogen availability. However, it is not clear whether an alteration in autophagy was responsible for the observed phenotypes, as cytokinin led to a decrease in the number of typical autophagosomes seen, but an increase in larger GFP-containing structures near the vasculature [40]. The relationship of these structures to the autophagy process is an important area for future investigation.

Another candidate for hormonal regulation of autophagy is abscisic acid (ABA), as ABA controls the response of plants to various abiotic stresses [43]. While a direct effect of ABA on autophagy has not been shown, interactions of ABA signaling with the TOR pathway, a potential regulator of autophagy (see below) have been observed. Increases in TOR expression lead to increased sensitivity of seedlings to ABA, and ABA may cause a decrease in TOR expression or activity [44]. The TOR pathway also affects the response of plants to osmotic stress [44,45]; again, whether this is related to autophagy or to an autophagy-independent function of the TOR signaling pathway remains to be seen.

3. Autophagy during developmentally regulated programmed cell death

The lack of major developmental phenotypes of *Arabidopsis* autophagy mutants [16,17] suggests that autophagy does not play a prominent role in most developmental processes, at least in this species. However, there is evidence for more subtle roles for autophagy in development, both in *Arabidopsis* and in other plant species.

An intriguing role for autophagy in the regulation of floret development, and therefore seed production, in wheat has emerged recently. In wheat, the spikelet meristem differentiates to produce up to twelve floret primordia, but these do not all develop into fertile florets [46]. Growth of wheat in long-day conditions was found to accelerate the transition from vegetative to reproductive development, that in turn reduced the number of fertile florets formed [29]. Examination of the aborted florets revealed evidence of programmed cell death in the ovaries, with morphological indications of autophagy. Electron microscopy studies showed double-membrane autophagosomes which entered the vacuole, and an increase in vacuolar size. The

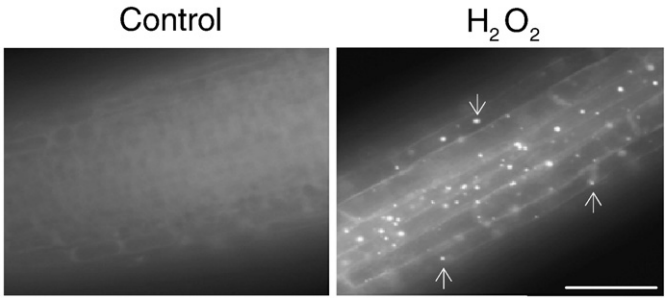

Fig. 2. Activation of autophagy by oxidative stress in *Arabidopsis* roots. Seven-day-old *Arabidopsis* seedlings were incubated under control conditions (left panel) or in the presence of 10 mM hydrogen peroxide (right panel) for 6 h to induce oxidative damage, followed by staining with monodansylcadaverine to label autophagosomes [37]. Roots were observed by fluorescence microscopy; labeled autophagosomes are visible as brightly fluorescent punctate structures (arrows). Scale bar = 50 μm. Modified from Xiong et al. [33] (www.plantphysiol.org). Copyright American Society of Plant Biologists.

expression of several autophagy-related genes, along with some proteases and cell death-associated genes, increased in the aborted florets, suggesting that autophagy occurs during, and potentially is responsible for, the cell death process.

These data raise the question of which pathways regulate the activation of the cell death process under long days, leading to reduced yield. Metabolite profiling studies demonstrated that the concentration of soluble carbohydrates decreased in florets before anthesis, and that this decrease occurred earlier under long day conditions. As nutrient deprivation is known to induce autophagy [6,7,47], one possibility is that induction of autophagy by sugar starvation in the ovaries leads to their death. Significantly, sucrose feeding led to an increase in the number of fertile florets [29], suggesting that autophagy may act as a regulator of fecundity in wheat.

Similar mechanisms may regulate cell death during petal senescence. Autophagy is responsible for the degradation of cellular components in the vacuole during petal senescence, although it is still not clear whether this is the actual cause of cell death or a mechanism for recycling material prior to death of the cell. Typical morphological indicators of autophagy are seen, including vesicles and cytoplasmic material inside the vacuole, an increase in vacuolar size and a loss of cytoplasmic volume and organelle content [48]. The tonoplast then collapses and cell death occurs. This process is regulated by sugar levels; it is possible that in some species, as proposed during wheat floret abortion, sugar starvation causes induction of autophagy, which in turn leads to cell death [49]. This is supported by measurements of ATP in flowers, showing that ATP is depleted in petals after opening, whereas exogenous sucrose maintains ATP levels and prevents or delays senescence [50].

The formation of xylem tracheary elements requires the large-scale degradation of cellular components prior to and during programmed cell death to produce a hollow tube. As xylogenesis is initiated, cytoplasmic material is taken up into the vacuole for degradation; later, the vacuole collapses and lyses, with complete degradation of the cell contents [51]. Even though autophagy genes are not upregulated during xylogenesis [52], this uptake of material into the vacuole most likely occurs via an autophagic process. Whether autophagy is directly responsible for cell death, however, is unknown. Two xylem-specific vacuolar cysteine proteases were identified as important for the clearance of cellular contents during xylogenesis [53,54]. Plants lacking these proteases accumulated undigested cell contents in the vacuole before lysis, which were still visible in the cell after vacuole lysis, suggesting that autophagic degradation is important in the removal and recycling of cytoplasmic contents in the formation of xylem [54].

Other types of programmed cell death in plants have been suggested by histological criteria to involve autophagy, although most are still awaiting molecular confirmation. For example, in the digitalis floral nectary, cell death begins at the onset of nectar secretion, with cell contents taken up into the vacuole for degradation [55]. The formation of sieve elements in wheat phloem involves a selective autophagy-like transfer of certain cytoplasmic components into the vacuole prior to eventual vacuole rupture [56], similar to the process occurring during xylem formation. In the case of sieve elements, it has been suggested that this process ceases just prior to actual death of the cell. In barley, programmed cell death occurs during caryopsis development and requires a cysteine protease with caspase-like activity that localizes to autophagosomes [57]. The precise role of autophagy, and whether these cell death pathways require the classical autophagy genes, remains to be seen.

4. Role of autophagy in pathogen immune responses

One of the primary mechanisms for defense against pathogens in plants is the hypersensitive response (HR), in which localized cell death in the infected plant cells restricts replication of the pathogen and movement out of the initial infection site, and thus controls spread of the disease throughout the plant [58]. Liu et al. used virus-induced gene silencing in *Nicotiana benthamiana* to identify genes required for a normal HR to infection with Tobacco Mosaic Virus, and identified the putative autophagy gene *ATG6*/*BECLIN1* as required for regulation of the response [23]. In the *ATG6* silenced plants, the cell death caused by Tobacco Mosaic Virus infection spread from the infection site throughout the leaf, and even to upper uninoculated leaves, although the virus itself did not move. It was therefore concluded that ATG6, or an ATG6-regulated process, restricts cell death during the HR. In control plants, but not silenced plants, autophagy was induced at the infection site, in the cells around the infection site and also in upper leaves, suggesting that the autophagy process may control the spread of cell death. This is further supported by the similar cell death phenotypes seen when additional autophagy-related genes were silenced [23]. In addition to viral infection, ATG6 is also required for restriction of cell death during fungal infection in tobacco [23] and bacterial infection in both tobacco and *Arabidopsis* [23,24], suggesting that this is a general mechanism for regulating programmed cell death in response to pathogens.

Interestingly, in addition to a role in the HR in resistant plants, *Arabidopsis* ATG6 also seems to be involved in controlling disease-associated cell death in susceptible plants. As complete knockouts in the *ATG6* gene are lethal in *Arabidopsis* [59–61], most likely due to functions of ATG6 that are unrelated to autophagy, antisense expression was used to generate plants with reduced *ATG6* expression, which showed typical autophagy phenotypes [24]. Upon infection with a disease-causing bacterial strain, in control plants disease-associated cell death was found only at the infection site under the experimental conditions used, whereas cell death spread in the antisense plants. The antisense plants also had increased numbers of bacteria at early time points, suggesting that ATG6, and possibly autophagy, limits pathogen growth during the initial stages of infection [24]. Reports that the *Arabidopsis* vacuolar protease VPE-γ has caspase-like activity that is required for appropriate induction of programmed cell death during the HR also supports a role for the vacuole in this process, although a relationship to autophagy has not been established [62–64].

These data bring up an intriguing possibility of modification of the autophagy pathway to improve disease resistance. As autophagy seems to play a role in the regulation of a wide range of pathogen responses, including innate immunity to viruses, fungi and bacteria and pathogen replication and spread upon infection [23,24], modulation of the timing or extent of autophagy induction could provide a broad defense against infection and disease. The signaling pathway that causes activation of autophagy in response to pathogens is unknown; the ability to alter this pathway therefore awaits the identification of the signal perception and transduction components involved.

5. Does the TOR pathway regulate autophagy in plant cells?

Despite significant progress in understanding the functions and mechanism of autophagy in plant cells, little is known about the signaling components that regulate autophagy. In both animals and yeast, the TOR (Target Of Rapamycin) protein kinase is a negative regulator of autophagy, and more broadly regulates multiple cellular processes, including growth and protein synthesis, in response to nutrient and growth factor availability [65–68]. The availability of a specific inhibitor for TOR, rapamycin, has aided in its functional characterization in numerous species; however, *Arabidopsis* and a number of other plant species appear insensitive to this inhibitor [45,69], which has impeded analysis of the TOR pathway in plants.

A gene encoding a homolog of TOR in yeast and animals was initially discovered in *Arabidopsis*, indicating that despite the insensitivity of this species to rapamycin, the TOR pathway does exist in plant cells [69] (Fig. 3). The *Arabidopsis TOR* gene is expressed

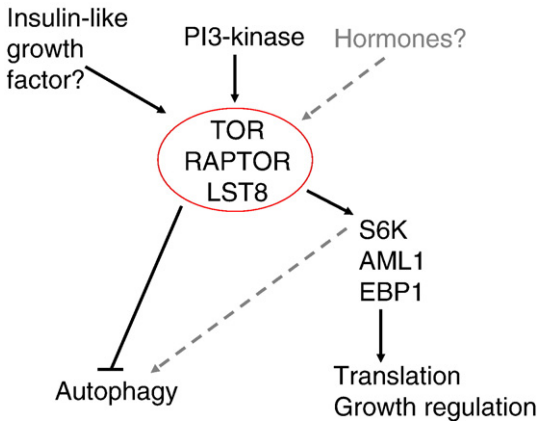

Fig. 3. Potential TOR signaling pathways in plants. TOR kinase is a putative regulator of autophagy and exists in a complex containing RAPTOR and LST8. S6K, AML1 and EBP1 are potential TOR substrates that may activate protein synthesis and/or growth, whereas the TOR complex is predicted to negatively regulate autophagy. TOR may in turn be activated by PI3-kinase activity, hormones and a putative insulin-like factor.

in rapidly growing and dividing tissues such as embryos, floral buds and meristems, suggesting a role in control of growth. Analysis of the TOR protein level in germinating maize seeds revealed that the protein was undetectable in dry seeds but increased during germination, again consistent with a role in growth [70]. A T-DNA knockout mutant in *TOR* was found to be embryo lethal, with embryos arrested at an early stage of development, indicating an essential function in growth and development but precluding further analysis [69]. To circumvent this problem, transgenic *Arabidopsis* plants with increased or decreased *TOR* expression were generated [44]. Overexpression of *TOR* led to increased shoot and root growth, with an increase in the size of epidermal cells and in seed production, whereas reduced *TOR* expression had the opposite effect. An inducible RNAi system was used to silence *TOR* expression at different developmental stages. Upon silencing in older plants that had already initiated flowering, almost complete cessation of growth occurred and early senescence was observed. In younger seedlings, silencing led to developmental arrest, with no root development and loss of expansion of cotyledons and hypocotyls. A reduction in high molecular weight polysomes was found, suggesting a control of translation by TOR in *Arabidopsis*, as seen in other species [44,66].

Despite a number of reports identifying TOR in plants, there is still little direct evidence for regulation of autophagy by TOR in photosynthetic organisms. Perhaps the best indication comes from work with the green alga *Chlamydomonas reinhardtii*, which like animal and yeast cells is sensitive to rapamycin, most likely due to inhibition of the TOR signaling pathway. Rapamycin inhibited the growth of *Chlamydomonas* and caused increased vacuolation, a typical autophagy effect [71]. *Chlamydomonas* TOR is membrane associated and localizes at least partially to the endoplasmic reticulum or other microsomal structures, again consistent with a role in autophagy initiation in addition to growth regulation [72].

6. Identification of additional components of the TOR complex

Yeast and animal TOR proteins are components of a complex that includes Raptor and Lst8 [73,74]. The function of Raptor appears to be to bind TOR substrates and present them to TOR for phosphorylation [75]. *Arabidopsis* contains two Raptor-like genes, *AtRAPTOR1A* and *1B*, which are expressed in growing tissues throughout the plant, although with *AtRAPTOR1B* expressed at a much higher level than *AtRAPTOR1A* [76,77]. AtRAPTOR1B was co-expressed as a GST fusion protein in tobacco leaves with the *Arabidopsis* TOR HEAT domain (named for the proteins in which it was first discovered; *h*untingtin, *e*longation factor 3, PR65/*A* subunit of protein phosphatase 2A, *T*OR) fused to GFP and interaction between the two proteins demonstrated by co-immunoprecipitation [45], suggesting that AtRAPTOR1B is a component of the *Arabidopsis* TOR complex. Knockout mutants in the *AtRAPTOR1A* and *1B* genes have been isolated, and no phenotype was found for *Atraptor1A* mutants. In contrast, knockouts in *AtRAPTOR1B* have been described either as stunted and developmentally delayed, with defects in root growth due to defective production of cells from the root meristem [76], or as embryo-lethal, with seed abortion and arrest of embryo growth at a very early pre-globular stage [77]. The basis for these extreme differences reported in mutant phenotype is unclear, as both are apparently null mutants. Anderson et al. were even able to generate *Atraptor1A*/*1B* double mutants, which arrested at the early seedling stage [76]. The addition of 1% sucrose to the growth medium partially rescued this phenotype, supporting the idea that the phenotypic differences could be due to differences in growth conditions of the mutant plants.

An LST8 homolog was identified in *Chlamydomonas* and shown to be present in a large complex along with TOR. LST8 co-localized with TOR to endoplasmic reticulum membranes, and was shown to interact with the TOR kinase domain. Complementation of a yeast *lst8* mutant with the *Chlamydomonas* gene indicated a conserved function of the protein across species [72].

7. Potential TOR substrates

Several potential plant TOR substrates have been proposed, primarily by analogy with substrates from other species. Mei2 is a meiosis signaling molecule that is a putative substrate of TOR in the fission yeast *S. pombe* [78]. A *Mei2*-like gene from *Arabidopsis* (*AML1*) is able to complement an *S. pombe mei2* mutant, suggesting functional conservation between the two species [79]. A yeast two-hybrid assay demonstrated interaction between AML1 and AtRAPTOR1B [80], which could potentially recruit AML1 to the TOR complex as a substrate for phosphorylation. *Arabidopsis* contains five *Mei2*-related genes, *AML1-5*, and surprisingly, knockout mutants in each gene gave a similar early flowering phenotype, with multiple knockouts also giving the same phenotype as singles. It was suggested that rather than the phenotype being caused by loss of expression of the appropriate gene, truncated proteins may be generated in the mutants that have a dominant negative effect, thus producing the same phenotype in each case; further analysis is needed to confirm this interpretation [80].

A second proposed target of TOR in *Arabidopsis* is EBP1 (ErbB-3 EGF Receptor Binding Protein), a regulator of ribosome assembly and function [81]. Alterations in the expression level of *EBP1* in transgenic *Arabidopsis* plants had the same effect as altering *TOR* expression, and the expression of the *EBP1* gene itself was altered in plants with increased or reduced *TOR* expression [44,81]. It was suggested that EBP1 is therefore a downstream target of TOR, although no direct evidence for this is yet available [44].

One of the best characterized TOR substrates is ribosomal protein S6 kinase (S6K), a regulator of translation [82]. Maize S6K increases in activity during seed germination, and this increase correlates with an increase in phosphorylation of the S6K protein [83]. *Arabidopsis* S6K physically interacts with AtRAPTOR, suggesting that it may be a direct TOR substrate [45].

The downstream events leading to activation of autophagy in plants have not yet been elucidated. In yeast, an ATG1/ATG13 complex regulates autophagy downstream of TOR. ATG1 is a protein kinase whose activity increases in response to starvation or rapamycin, thus causing activation of autophagy. ATG1 is activated by ATG13, and this is dependent on the phosphorylation status of ATG13 [84]. Homologs of ATG1 and ATG13 exist in plants, and are predicted to regulate autophagy in a similar way to the yeast proteins [15,32]. In addition, other signaling pathways may converge with the TOR pathway to coordinate nutrient responses. For example, the SnRK1 kinase family

activates many genes known to be involved in starvation responses, including several autophagy genes [85,86]. It is likely that a number of pathways cooperate in regulating autophagy in response to changing nutrient and environmental conditions.

8. Upstream regulators of TOR activity

Insulin is a major regulator of the TOR pathway in mammals [74]. While plants do not appear to have close homologs of insulin or components of the insulin-signaling pathway, a potential insulin-like protein was identified immunologically in maize tissues [87]. While this is an intriguing observation, whether this protein is truly analogous to mammalian insulin is unclear at this point. Incubation of the protein with germinating maize seeds led to faster germination and increased growth rate, with increased ribosome phosphorylation and ribosomal protein synthesis. This increase was correlated with, and potentially caused by, increased S6K phosphorylation and activity [83,88]. The activity of the putative insulin-like protein was antagonized by rapamycin, suggesting that this effect is due to the TOR pathway, and also that unlike *Arabidopsis*, maize TOR is sensitive to this inhibitor [83,87,88].

In animal cells, autophagy is inhibited by 3-methyladenine (3-MA) via inhibition of PI3-kinase activity [89–91]. The PI3-kinase inhibitors 3-MA, wortmannin and LY294002 all blocked sucrose starvation-induced autophagy and protein degradation in tobacco cell cultures [92], strongly suggesting that PI3-kinase activity is required for autophagy in plant cells. The inhibitors blocked autophagosome accumulation at a very early stage, with no intermediates in autophagosome formation visible, indicating that PI3-kinase may be involved in regulation of autophagy. Whether this PI3-kinase activity functions upstream or downstream of TOR remains to be seen, and may be dependent on the species under study [93].

9. Conclusions

It is now clear from morphological and genetic studies that autophagy functions in plants as a nutrient recycling pathway as it does in other organisms. It has also been shown that plants contain homologs of most of the yeast autophagy genes and that their functions are conserved between species [15,31]. However, current research is revealing a number of additional functions of plant autophagy, including responses to abiotic stress conditions and regulation of programmed cell death in pathways ranging from pathogen defense to flower and vascular development [23,29,33,40,54]. Despite this wide variety of conditions in which autophagy is important, little is known about how the pathway is regulated by these conditions. Recent studies have indicated the presence of a functional TOR pathway in plants [44,45,69,70,88], a key pathway regulating autophagy in animals and yeast, and future research should provide insight into how this pathway may integrate environmental and hormonal signals to activate autophagy in the appropriate conditions, cell types and stages of development.

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