Analysis of the function and the regulation of autophagy in Arabidopsis thaliana

Yimo Liu
Iowa State University

Follow this and additional works at: http://lib.dr.iastate.edu/etd

Part of the Biology Commons, Cell Biology Commons, and the Plant Biology Commons

Recommended Citation
Liu, Yimo, "Analysis of the function and the regulation of autophagy in Arabidopsis thaliana" (2012). Graduate Theses and Dissertations. 12385.
http://lib.dr.iastate.edu/etd/12385
Analysis of the function and the regulation of autophagy in *Arabidopsis thaliana*

by

Yimo Liu

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Major: Genetics

Program of Study Committee
Diane C. Bassham, Major Professor
Gustavo MacIntosh
David J. Oliver
Steven R. Rodermel
Yanhai Yin

Iowa State University
Ames, Iowa
2012
Copyright © Yimo Liu, 2012. All rights reserved.
# TABLE OF CONTENTS

ABSTRACT iv

CHAPTER 1 GENERAL INTRODUCTION 1

1.1 Autophagy: pathways for self-eating in plant cells 1

1.1.1 Abstract 1

1.1.2 Introduction 2

1.1.3 Machinery and mechanisms of autophagy in plants 3

1.1.4 Functions of autophagy during abiotic Stress 8

1.1.5 Functions of autophagy during development 9

1.1.6 Functions of autophagy during programmed cell death 11

1.1.7 Regulation of autophagy in plants 15

1.1.8 Evidence for selective autophagy 19

1.1.9 Summary points 22

1.1.10 Future issues 22

1.1.11 Mini-glossary 23

1.1.12 Acknowledgments 24

1.1.13 Literature cited 24

1.1.14 Figures and tables 33

1.2 Dissertation organization 39

CHAPTER 2 AUTOPHAGY IS REQUIRED FOR TOLERANCE OF DROUGHT AND SALT STRESS IN PLANTS 40

2.1 Abstract 40

2.2 Introduction 41

2.3 Results 43

2.4 Discussion 49

2.5 Methods 52

2.6 Acknowledgements 55

2.7 References 55

2.8 Figures and tables 60
CHAPTER 3 TOR IS A NEGATIVE REGULATOR OF AUTOPHAGY IN ARABIDOPSIS THALIANA

3.1 Abstract 69
3.2 Introduction 70
3.3 Results 72
3.4 Discussion 80
3.5 Materials and methods 83
3.6 Acknowledgements 85
3.7 References 85
3.8 Figures and tables 90

CHAPTER 4 DEGRADATION OF THE ENDOPLASMIC RETICULUM BY AUTOPHAGY DURING ER STRESS IN ARABIDOPSIS THALIANA 97

4.1 Abstract 97
4.2 Introduction 98
4.3 Results 101
4.4 Discussion 112
4.5 Methods 116
4.6 Acknowledgements 120
4.7 References 120
4.8 Figures and tables 126

CHAPTER 5 CONCLUSIONS AND FUTURE WORK 140

5.1 General conclusions 140
5.2 Future directions 142
5.3 References 143
5.4 Figures and tables 145

ACKNOWLEDGEMENTS 146
ABSTRACT

Plants have developed sophisticated mechanisms to survive under adverse growth conditions. Autophagy is activated in response to multiple abiotic stresses, pathogen infection and senescence in plants. Upon induction of autophagy, portions of cytoplasm are engulfed by double membrane structures termed autophagosomes and are delivered to vacuole for degradation. Over the past decade, the identification of many autophagy-related genes in plants has greatly enhanced our molecular and physiological understanding of plant autophagy. However, the upstream regulatory components in the autophagy pathway and the function of autophagy under various stresses are still unclear. This thesis summarizes my efforts in studying the function and the regulatory pathways of autophagy in Arabidopsis thaliana.

Previous research indicates that autophagy is required for plant tolerance of nutrient and oxidative stress. To further explore the function of autophagy in other abiotic stresses, the induction of autophagy was investigated in response to high salinity, osmotic and ER stresses. Our results showed that autophagy is induced by high salinity and osmotic stresses in Arabidopsis. Autophagy-defective plants are more sensitive to salt and drought stresses than wild-type plants, indicating the participation of autophagy in the response to these stresses. The ER stress-inducers tunicamycin and dithiothreitol (DTT) both caused rapid activation of autophagy in Arabidopsis. Microscopy analysis suggests a role for autophagy in the transport of ER fragments to the vacuole for degradation during ER stress in plants. In addition, one of the ER stress sensors, IRE1b, was found to be required for ER stress-induced autophagy. Interestingly, autophagy was found to be regulated by different pathways during different abiotic stresses. In nutrient starvation and salt stress, autophagy is regulated by an NADPH oxidase-dependent pathway, whereas in osmotic and ER stresses, autophagy is regulated by an NADPH oxidase-independent pathway.

Target of rapamycin (TOR) kinase is the central controller of many cellular functions in eukaryotic cells in response to stress and nutrient deprivation. In yeast and animals, TOR is
shown to negatively control the autophagy pathway, whereas little is known about its role in autophagy in plants. To obtain more knowledge on the molecular function of TOR in autophagy, RNA interference (RNAi)-TOR transgenic plants with decreased expression of TOR were generated. These plants showed constitutive autophagy even under favorable growth conditions, indicating that TOR is also a negative regulator of autophagy in Arabidopsis.

Together our results revealed that autophagy is activated in response to salt, osmotic and ER stresses, which suggests that autophagy is a general defense mechanism to protect plants during unfavorable environments. We also provide evidence that TOR negatively controls autophagy in plants, increasing our understanding of the regulatory mechanism of autophagy in plants.
CHAPTER 1
GENERAL INTRODUCTION

1.1 Autophagy: pathways for self-eating in plant cells

A paper accepted by Annual Review of Plant Biology*
Yimo Liu†, Diane C Bassham¹,²†

1. Department of Genetics, Development and Cell Biology and Interdepartmental Genetics Program, Iowa State University, Ames, Iowa 50011
2. Plant Sciences Institute, Iowa State University, Ames, Iowa 50011

1.1.1 Abstract

Plants have developed sophisticated mechanisms to survive when in unfavorable environments. Autophagy is a macromolecule degradation pathway that recycles damaged or unwanted cell materials upon encountering stress conditions or during specific developmental processes. Over the past decade our molecular and physiological understanding of plant autophagy has greatly increased. Most of the essential machinery required for autophagy seems to be conserved from yeast to plants. Plant autophagy has been shown to function in various stress responses, pathogen defense and senescence. Some of its potential upstream regulators have also been identified. Here, we describe recent advances in our understanding of autophagy in plants, discuss areas of controversy and highlight potential future directions in autophagy research.

Key words: abiotic stress, programmed cell death, pathogen, TOR, senescence

† Primary author
‡ Author for correspondence
1.1.2 Introduction

Autophagy (meaning “self-eating”) is a macromolecule degradation process in which cells recycle cytoplasmic contents when under stress conditions or during developmental transitions. The basic autophagy process is conserved among eukaryotes from yeast to animals and plants (9, 82, 135). Several types of autophagy have been described in many species, including microautophagy (84), macroautophagy (135), chaperone-mediated autophagy (92), and organelle-specific autophagy (103). In plants, microautophagy and macroautophagy have been shown to occur (11). Microautophagy involves the formation of a small intravacuolar vesicle called an autophagic body by invagination of the tonoplast, thus engulfing cytoplasmic components, whereas in macroautophagy, cytoplasmic autophagosomes enclose components to be degraded (11). Here, we focus on the macroautophagy pathway in plants, hereafter referred to as autophagy.

The principal characteristic of autophagy is the formation of double-membrane structures called autophagosomes (Figure 1.1). Upon induction of autophagy, an autophagosome forms around the material that is destined for degradation, and the autophagosome delivers this cargo to the vacuole. The outer membrane of the autophagosome fuses with the vacuole membrane, after which vacuolar hydrolases degrade both the cargo and the inner membrane in the vacuole.

Although plant autophagy was discovered several decades ago (80, 81, 120), we have only recently begun to understand its molecular mechanism. Most genes functioning in the autophagy pathway were first identified via mutagenesis studies in yeast. More than 30 autophagy-related genes have been identified in yeast; these genes can be divided into several functional groups (136): the Atg1-Atg13 kinase complex, Atg9 and associated proteins, a phosphatidylinositol 3-kinase (PtdIns3K) complex, and two ubiquitin-like conjugation systems. These studies in yeast have greatly facilitated the identification of homologous genes in plants that are required for autophagy and have provided direction for investigating their molecular functions.
In animals, autophagy is implicated in health and disease processes such as cancer, neurodegeneration, aging, and longevity (136); in plants, it is associated with a variety of stresses, pathogen infection, and senescence (9, 10, 38). Under normal conditions, basal autophagy functions as a housekeeping process to clear damaged or unwanted cytoplasmic contents, whereas under certain stresses, autophagy is upregulated (47, 108, 133). Autophagy-defective plants usually senesce earlier and are more susceptible to stress conditions compared with wild-type plants (Table 1.1). Several markers have been developed to study autophagy in plants, most commonly green fluorescent protein (GFP)--ATG8s (19, 117, 142) and monodansylcadaverine (MDC) staining (19). These markers allow the rapid and straightforward detection of autophagy occurrence in plant cells by the specific labeling of autophagosomes. In this review, we summarize recent advances in our understanding of plant autophagy, including the essential machinery, regulation, and physiological roles, and briefly discuss the emerging evidence for selective autophagy.

1.1.3 Machinery and mechanisms of autophagy in plants

Ubiquitin-like conjugation systems
The yeast autophagy pathway requires two ubiquitin-like conjugation systems, which in turn involve two ubiquitin-like proteins, Atg8 and Atg12 (for reviews on mechanistic aspects, see 90, 135). After Atg8 is synthesized, its C-terminus is first cleaved by a cysteine protease, Atg4, to expose a glycine residue. The exposed glycine is bound by an E1-like enzyme, Atg7, and the Atg8 is then transferred to an E2-like enzyme, Atg3. Finally, the Atg8 is conjugated to the membrane lipid phosphatidylethanolamine (PE). This Atg8-PE conjugation is reversible, as the protease Atg4 also cleaves Atg8 from PE; the released Atg8 can thus be recycled. Another ubiquitin-like protein, Atg12, is also activated by Atg7; it is then transferred to an E2-like enzyme, Atg10, and finally conjugated to Atg5. The Atg12-Atg5 conjugate further interacts with a coiled-coil protein, Atg16, to form a tetrameric Atg12-Atg5·Atg16 complex (in which hyphens indicate covalent bonds whereas the dot represents a non-covalent interaction) via Atg16 oligomerization. This complex is also essential for autophagy. Atg8-PE conjugates and Atg12-Atg5·Atg16 complexes reside on the
preautophagosomal structure (PAS), and upon induction of autophagy, both of them localize
to the expanding phagophore. Atg8-PE conjugates show an equal localization on both the
inner and outer autophagosome membrane, whereas the Atg12-Atg5-Atg16 complexes
mainly associate with the outer membrane (135).

In *Arabidopsis*, all of the counterparts of the two yeast conjugation systems are also well
conserved (Figure 1.2). In contrast to yeast, which has a single *ATG8*, *ATG4* and *ATG12*
gene, *Arabidopsis* contains nine members of the *AtATG8* family (*AtATG8a--AtATG8i*), two
members of the *AtATG4* family (*AtATG4a, AtATG4b*), and two members of the *AtATG12*
family (*AtATG12a, AtATG12b*) (27, 34).

Both *AtATG4* s are ubiquitously expressed in plants, and their expression levels are elevated
after nitrogen starvation (142). Phenotypic analysis of an *Atatg4a4b* double mutant showed
that it displays the phenotypes of early senescence and reduced silique production that are
typical of autophagy defects (142). There are contradictory reports on whether the growth of
primary roots and lateral roots in the *Atatg4a4b* double mutant are arrested under nitrogen-
limited conditions (16, 142). As in yeast, *AtATG4* s function as proteases to process the C-
terminus of *AtATG8* s, and autophagosomes cannot form in the *Atatg4a4b* double mutant
(142). These findings indicate that the *ATG4* s are essential for plant autophagy and that
they function similarly to *ATG4* in yeast.

Under normal growth conditions, all of the nine *AtATG8* s are expressed throughout the plant,
although different members show distinct expression patterns (108, 142), implying that each
member may have a distinct function during development or under various stress conditions.
Several independent studies have demonstrated the existence of the *AtATG8* ubiquitin-like
conjugation system in *Arabidopsis* (16, 27, 29, 117, 142). The C-terminus of the *AtATG8* s is
cleaved by *AtATG4*; bound to an E1-like enzyme, *AtATG7*; transferred to an E2-like
enzyme, *AtATG3*; and finally conjugated to PE (Figure 1.2). ATG8 orthologs have also been
identified in *Chlamydomonas* (96), rice (17, 112), and maize (17).
In mammals, ATG8s are divided into two subfamilies according to their protein sequence similarity: the LC3 subfamily (four members) and the GABARAP/GATE-16 subfamily (four members). The LC3 subfamily is involved in elongation of the phagophore membrane, whereas the GABARAP/GATE-16 subfamily is involved in autophagosome maturation (129). It has been suggested that in Arabidopsis, AtATG8s bind to microtubules, indicating the possible involvement of the cytoskeleton in plant autophagy (57). In one study, overexpression of a GFP-AtATG8f-HA fusion protein in Arabidopsis plants enhanced growth and altered stress responses, and cytokinin-mediated regulation of root architecture and root--shoot communication were affected (109); this suggests that AtATG8s may have a physiological role in responding to hormones and abiotic stresses. Recently, several studies showed that in yeast and animals, ATG8/LC3 may be crucial during selective autophagy in recognition of a specific protein motif, the ATG8-interacting motif (AIM) (89). Atg19 and p62 use the same WXXL motif to interact with ATG8/LC3 family members (88). NBR1 (neighbor of BRCA1 gene), Atg32, and Nix also use a similar motif to bind to the ATG8/LC3 family (89). So far, in plants, two proteins have been found to use an AIM to interact with ATG8s: AtNBR1 and AtTSPO (tryptophan-rich sensory protein) (116, 122). AtNBR1 is a selective autophagic substrate that binds to AtATG8s via an AIM motif (116). The stress-induced AtTSPO is degraded by the autophagy pathway, and the AIM-like motif is required for this process (122).

In Arabidopsis, there are two members of the AtATG12 gene family: AtATG12a and AtATG12b (16, 27, 34). These two genes share high amino acid similarities (95%) and have functional overlap; however, AtATG12b is more important during basal autophagy, whereas AtATG12a is more important during induced autophagy (16). All of the components of the AtATG12 conjugation system have been characterized (16, 27, 97, 117). Atatg5, Atatg7, Atatg10, and Atatg12a12b mutants are all autophagy-defective and display the typical phenotypes of early senescence and hypersensitivity to nutrient-limited conditions (16, 27, 97, 115, 117). Just as in yeast, the AtATG12-AtATG5 conjugate functions in the formation of the AtATG8-PE conjugate (16, 29, 33) (Figure 1.2). In rice, two ATG10 genes have been identified, OsATG10a and OsATG10b (107), which play an important role in autophagosome
formation and in survival during oxidative stress (107). Maize ZmATG12 has also been identified and found to interact with ZmATG7 (17). These findings suggest that the ATG12 conjugation system is conserved in plants and is essential for the plant autophagy pathway.

**Atg9 cycling system**

During the formation of autophagosomes, a major puzzle is the source of the lipid membrane. In yeast, the phagophore is thought to be generated at a single perivacuolar site, the PAS (114). An integral membrane protein, Atg9, has been proposed to deliver lipid to the forming autophagosomes (39, 131). Atg9 localizes to the PAS and several non-PAS punctate structures; it is postulated that Atg9 cycles between the PAS and non-PAS structures. In yeast, the Atg9 non-PAS puncta apparently consist of tubulovesicular clusters adjacent to mitochondria, and the autophagosome membrane forms de novo (79). In contrast, mammalian cells may possess multiple PASs, and mAtg9 localizes to a juxtanuclear region corresponding to the *trans*-Golgi network and late endosomes (145). It has been proposed that in animals, the autophagosome membrane derives from several sources, including the endoplasmic reticulum (ER) (37, 139), mitochondria (32), and plasma membrane (100). In plants, however, the membrane origin is not known.

In yeast, the movement of Atg9 from non-PAS puncta to the PAS requires the function of Atg11, Atg23, and Atg27 (131, 135). The movement of Atg9 from the PAS to non-PAS puncta involves the Atg1-Atg13 kinase complex, Atg2, Atg18, and PtdIns3Kcomplex I (131). Defects in any of these components lead to the accumulation of Atg9 at the PAS. Atg2 and Atg18 are peripheral membrane proteins, both of which can interact with Atg9 (131, 135). Atg18 can bind to phosphatidylinositol 3-phosphate [PtdIns(3)P] and PtdIns (3,5)P2, and binding to PtdIns (3)P protects Atg8-PE from unregulated cleavage by Atg4 and is required for autophagy (85).

In *Arabidopsis*, although homologs of *ATG1*, *ATG2*, *ATG9*, *ATG13*, and *ATG18* have been found (6), of these only the *ATG2*, *ATG9*, and *ATG18* homologs have been characterized in detail. *Arabidopsis* has a single *AtATG9* gene and a single *AtATG2* gene, which are expressed
ubiquitously throughout the plant (34, 125, 143). Both \textit{Atatg}9 and \textit{Atatg}2 knockout mutants display typical autophagy-defective phenotypes during senescence and stress conditions (34, 125, 143). There are eight members in the \textit{AtATG18} gene family (\textit{AtATG18a}--\textit{AtATG18h}) (34, 132); each member has a different expression pattern, and only one (\textit{AtATG18a}) shows an increased transcript level in starvation conditions and during senescence (132). \textit{AtATG18a} expression is also upregulated and is required for autophagy during oxidative, salt, and osmotic stresses (74, 133, 134). RNA interference (RNAi)--\textit{AtATG18a} plants are autophagy-defective and display a typical autophagy phenotype, and are more sensitive to various stress conditions (Figure 1.3) (74, 132--134). The identification of these counterparts to yeast genes in \textit{Arabidopsis} may suggest conserved roles. However, their subcellular localizations and specific functions during autophagosome biogenesis remain to be determined.

\textbf{PtdIns3K complex}

In yeast, a PtdIns3K complex is required for autophagy and localizes to the PAS (135). It includes a class III PtdIns3K, Vps34; a serine/threonine kinase, Vps15; Vps30/Atg6; and Atg14. Vps15 is required for the membrane association of Vps34. Atg14 is thought to connect Vps34 and Vps30/Atg6 (131, 135), whereas the function of Vps30/Atg6 is not clear. The PtdIns3K complex has been postulated to recruit PtdIns (3)P binding proteins, including Atg18, to the PAS (135).

In \textit{Arabidopsis}, only one PtdIns3Khas been identified, \textit{AtVPS34} (130), which is an essential protein (130). It has been reported to have diverse physiological functions in plants (53, 64, 69), and genetic transmission analysis showed that \textit{AtVPS34} is essential for pollen development and vacuole reorganization (65). \textit{ATG6} is also a single gene in \textit{Arabidopsis}, and it is expressed ubiquitously throughout the plant (28, 98). \textit{AtATG6} colocalizes with \textit{AtATG8s} to autophagosomes (28, 36). Several independent reports have shown that \textit{AtATG6} gene function is essential for pollen germination, although this phenotype may be unrelated to its role in autophagy (28, 36, 98). Plants with disrupted \textit{AtATG6} have increased anthocyanin production, short roots, early leaf senescence, dwarfism, fewer flowers, and low fertility (36, 94, 98). Also, \textit{AtATG6} antisense plants fail to limit the pathogen-associated cell
death response (94). In tobacco (*Nicotiana benthamiana*), *NbATG6* is required for pathogen-induced autophagy and regulation of programmed cell death (PCD) (73). There is one *VPS15* homolog in the *Arabidopsis* genome (6), but its function has not been studied in detail; *ATG14* appears to be missing. Moreover, how these genes are coordinated to regulate autophagy in plants is still unclear.

**Vti12 SNARE**

After the double-membrane autophagosome forms, it is delivered to the vacuole for further degradation. This process requires the fusion of the autophagosome membrane with the vacuole membrane. Several components of the SNARE (soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptor) machinery are required for the fusion process and autophagosome membrane expansion in yeast (86, 135). Several homologs of the VTTI vesicle SNARE are present in *Arabidopsis* (105), and one of these homologs, VTI12, is thought to function during autophagy (113). A *vti12* mutant is more sensitive to starvation conditions and shows an accelerated senescence phenotype, suggesting a role in plant autophagy (113). The partner target SNAREs of VTI12 that may also function in autophagy are not known.

**1.1.4 Functions of autophagy during abiotic Stress**

The first and most common abiotic stress shown to induce autophagy was nutrient deprivation (27, 34, 97, 117, 132). Autophagy-defective plants display accelerated starvation-induced chlorosis, most likely because autophagy is required for nutrient remobilization during the starvation response. In recent years, autophagy has been shown to be a rather general response to a variety of abiotic stresses (9). When oxidative stress is introduced using *H*₂*O*₂ or methyl viologen, autophagy is quickly induced. The autophagy-defective RNAi-*AtATG18a* plants are more sensitive to methyl viologen treatment and accumulate higher levels of oxidized proteins (133, 134); similar phenomena have also been observed in a rice *Osatg10* mutant (107). These results suggest a role for autophagy in degrading oxidized proteins in plant cells. Autophagy is also required for plant tolerance to drought and salt
stresses (Figures 1.3 and 1.4) (74), implying that autophagy plays a role in removing damaged proteins or organelles during these stresses. AtTSPO, which is an abscisic acid--induced protein, has been shown to be degraded by the autophagy pathway, indicating the involvement of autophagy in responses to abscisic acid in plants (122).

1.1.5 Functions of autophagy during development

Although autophagy has been investigated most extensively during stress conditions, plants maintain a basal level of housekeeping autophagy even under favorable growth conditions (47, 108, 138). This basal autophagy may function to eliminate damaged proteins and organelles, which are continually generated under normal growth conditions (11). Almost all of the autophagy-defective mutants are able to complete their life cycles but have an early-senescence phenotype even under nutrient-rich conditions (27, 34, 94, 97, 98, 117, 132, 142), which suggests that autophagy has some function under these conditions. Because autophagy recycles cytoplasmic materials, it is understandable that it functions during senescence and germination, the two large-scale nutrient remobilization processes in the plant life cycle. About 80% of leaf nitrogen is contained in chloroplasts (78); during senescence, plants recycle nutrients from senescing leaf chloroplasts to the newly forming organs such as developing seeds (31). Small vesicles containing chloroplast stromal components [RuBisCO-containing bodies (RCBs); for more discussion, see Evidence for Selective Autophagy, below] have been shown to be degraded by the autophagy pathway (48, 49, 123, 124). In Arabidopsis, chloroplasts normally decrease in both size and number during senescence, which does not occur in an autophagy mutant (124). This raises an interesting question regarding the relationship between autophagy and chloroplast breakdown during leaf senescence. If autophagy is involved in chloroplast degradation, delayed senescence might be predicted if autophagic degradation is impaired. However, autophagy-defective mutants unexpectedly show early-senescence phenotypes, with accelerated loss of chlorophyll and chloroplast proteins. This indicates that an ATG gene--independent mechanism exists that is at least partially responsible for chloroplast recycling (11, 71)---for example, the action of chloroplast-localized proteases. One possibility is that autophagy is normally activated early
in the senescence pathway to begin the protein degradation process, leaving the photosynthetic machinery intact to continue photosynthesis. Additional catabolic pathways, including chloroplast-localized pathways for chlorophyll degradation, would be activated later in the senescence process (43). It is therefore hypothesized that when autophagy is blocked, the autophagy-independent pathways are activated prematurely, leading to early breakdown of chloroplast components and premature senescence.

To ensure seed viability, plants synthesize large amounts of seed storage proteins and deposit them into protein storage vacuoles (PSVs) during seed development. Upon seed germination, these proteins are degraded to support the growth of newly forming organs (46). In wheat, during seed development, the prolamin storage proteins are synthesized in the ER and form ER protein bodies; the direct transport of prolamins from ER to PSV involves a pathway that, under electron microscopy, resembles autophagy (70). In Vigna mungo, breakdown of starch granules during seed germination is also associated with the autophagy pathway (119). In Arabidopsis, several ATG genes are upregulated during seed maturation and desiccation (5), but no obvious defect in seed formation or germination has been observed in the ATG mutants under normal growth conditions. During salt stress, the germination of RNAi-AtATG18a seeds lags behind that of wild-type (WT) seeds (74), implying that autophagy may be involved in salt tolerance during seed germination. In maize aleurone cells, the prolamin storage protein zein was recently found to be delivered from ER to PSVs in atypical prevacuolar compartments. These zein-containing compartments have multilayered membranes and engulfed cytoplasmic materials, thus morphologically resembling autophagosomes (104). However, they are neither surrounded by a double membrane nor decorated by the Atg8 protein, suggesting that there is an atypical autophagy pathway to deliver storage proteins from ER to PSVs that is independent of ATG8 (104).

It has been previously suggested that autophagy plays a role in vacuole biogenesis (11, 80). However, ATG mutants do not have vacuole defects in either nutrient-limited or nutrient-rich conditions. Research in tobacco (BY-2) miniprotoplasts (protoplasts lacking the large central vacuole) demonstrated that the autophagy pathway involved in vacuole biogenesis is
mechanistically distinct from the stress-induced and basal autophagy observed throughout the plant growth cycle (137). In miniprotoplasts, after researchers used cysteine protease inhibitors to inhibit vacuolar protein degradation, cytoplasmic contents were detected in the newly generated vacuole, indicating the occurrence of autophagy during this process. However, inhibitors typically used to block stress-induced and basal autophagy did not affect the autophagy in miniprotoplast vacuole formation, suggesting that this is an atypical autophagy with a mechanism different than that of stress-induced and basal autophagy (137). In animals, alternative autophagy pathways that use only part of the canonical autophagy machinery to form functional autophagosomes have been described, including Atg5/Atg7-independent autophagy (87) and Beclin-1-independent autophagy (106). However, whether such noncanonical autophagy pathways exist in plants is still unknown.

1.1.6 Functions of autophagy during programmed cell death

In animals, PCD can be morphologically divided into three types: apoptosis, autophagic cell death, and necrosis (59). However, there is not an absolute distinction between the different forms of cell death, as several examples display mixed features (59). To further complicate analysis, in many cases that are defined as autophagic cell death, the studies show only that PCD occurred with concomitant activation of autophagy, rather than that the PCD process was carried out by autophagy (59). In plant cells, the typical animal apoptosis process does not seem to occur, because the presence of a cell wall prevents the dead cell from being engulfed by adjacent cells. Plant PCD seems to share some features with both apoptosis and autophagy in animals (for a more comprehensive review on plant PCD, see 63, 121). For example, the cell takes up organelles into the vacuole, the organelles are degraded, the vacuolar size increases, and eventually the vacuole lyses; these are all characteristics of autophagic cell death. However, the cell also undergoes chromatin condensation, nuclear fragmentation, and DNA laddering, which are characteristics of apoptotic cell death. In mammalian PCD, a group of cysteine proteases called caspases are important regulators of apoptosis (21). True caspase homologs have not been identified in plants (101), although several groups have found caspase-like activities. In barley, VEIDase was found to have a
caspase-like activity; it is localized to autophagosomes, linking the caspase activity to autophagic PCD (12). In Arabidopsis, type I metacaspases were also shown to control cell death (18). Collectively, known features of PCD in plants indicate that classification is not as clear as it is in animals. Plant PCD seems to have conserved functions but also unique characteristics. Research on plant PCD has mainly focused on two categories: PCD during normal development and PCD during the hypersensitive response (HR) triggered by pathogen infection. Evidence implies that autophagy plays critical roles in both processes.

**Functions of autophagy during developmental programmed cell death**

There are many well-known examples of PCD during various developmental stages (121), including xylem and phloem formation, senescence, shoot elimination, leaf shape formation, and pollen germination and tube growth. Morphological studies have shown that most of these processes involve the gradual disappearance of organelles and eventually the collapse of the tonoplast and plasma membrane, and it has been suggested that autophagy is responsible for the cell death (121). In wheat, accelerated plant development caused by long-day growth conditions can trigger developmentally generated sugar starvation, which in turn initiates autophagic cell death of florets and leads to decreased fertile floret numbers (30). The involvement of autophagy was also demonstrated by the analysis of PCD during xylem fiber maturation in Populus. Not only does this type of PCD morphologically resemble autophagic cell death, but several autophagy-related genes are upregulated (20).

Recently, one study (61) showed that autophagy also functions in xylem tracheary element (TE) differentiation in Arabidopsis. In this study, both protoxylem and metaxylem cell numbers decreased significantly in an Arabidopsis atg5 mutant compared with WT plants, suggesting that ATG5-dependent autophagy is involved in xylem development. LysoTracker Green staining showed that autophagy is induced during xylem differentiation. In addition, the study showed that a small GTP-binding protein, RabG3b, which was previously identified as a salicylic acid (SA)--responsive protein, is a positive regulator of autophagy during TE differentiation. These results demonstrate that RabG3b functions as a component of autophagy and regulates TE differentiation by activating the PCD process.
Functions of autophagy during pathogen infection

It has been suggested that autophagy can serve both a “prosurvival” and a “prodeath” role upon pathogen infection, depending on the type of pathogen, the type of immune factor involved, and the age of the plant (38, 42, 67, 68, 140, 144). Phytopathogens can be divided into two types based on their lifestyles: necrotrophic and biotrophic. Necrotrophic pathogens release toxins and lytic enzymes to kill the host cell, whereas biotrophic pathogens depend on their host cell to survive (67). In Arabidopsis, infection with the necrotrophic fungal pathogen Botrytis cinerea induces autophagy in both the infected and the surrounding areas (62). Compared with WT plants, autophagy-defective mutants are hypersensitive to Botrytis cinerea and Alternaria brassicicola: they have more dead cells, the degradation of certain proteins accelerates (62), and they develop spreading necrosis (68). Interestingly, the transcription factor WRKY33, which is important for plant resistance to necrotrophic pathogens, interacts with the autophagy protein ATG18a in the nucleus (62). These results clearly show that autophagy functions in a prosurvival role during necrotrophic pathogen infection.

The role of autophagy upon biotrophic pathogen infection is more complicated. When tobacco (Nicotiana benthamiana) plants are infected with tobacco mosaic virus, autophagy is induced in both the infected and the uninfected area (73). BECLIN1/ATG6/VPS30-silenced plants are capable of initiating HR-PCD; however, the PCD is unrestricted and spreads to healthy uninfected tissue and distal leaves (73). Similar results were later obtained in Arabidopsis ATG6 RNAi plants and atg5 knockout mutants (94, 143). After infection with an avirulent bacterial pathogen, Pseudomonas syringae pv. tomato (Pst) DC3000 (avrRPM1), the HR-PCD escapes from the infected area and spreads to adjacent healthy tissues (94, 143). These results suggest that autophagy functions to restrict the HR-PCD and prevent runaway cell death, thus serving a prosurvival role. However, another study has reported that cell death is suppressed and resistance is increased in atg7 and atg9 mutants infected with the avirulent bacterial strain Pst DC3000 (AvrRps4) (42). Similarly, the atg2 mutant displays enhanced powdery mildew resistance (125). Additional Arabidopsis autophagy-defective plants were recently tested for their immunity response to different types of pathogens (68);
compared with WT plants, the \textit{atg}-defective plants all showed enhanced resistance to the virulent bacterial strain \textit{Pst} DC3000 or to the avirulent \textit{Hyaloperonospora arabidopsidis}, possibly owing to an elevated SA level in the \textit{atg} mutant (discussed below) (42, 68). These results indicate that autophagy serves a prodeath role upon biotrophic pathogen infection. It has been suggested that the contradictory results during biotrophic pathogen infection are caused by the age differences in plants used in the experiments, which is related to SA signaling (see below). The spreading of the PCD was observed only in older leaves of older plants (7--8 weeks old), whereas no difference was observed in younger leaves or younger plants (4--5 weeks old) (38, 62, 143, 144).

The difference in autophagic response to necrotrophic and biotrophic pathogens may be due to the engagement of different plant hormones. The defense response to necrotrophic pathogens depends on jasmonic acid (JA) and ethylene signals, whereas the defense response to biotrophic pathogens involves the SA signal (52, 58, 111). Because the \textit{atg} mutants display altered immune response to both types of pathogens, several research groups have analyzed hormone levels and the expression of hormone-regulated genes during pathogen infection in both WT and autophagy-defective plants (62, 68, 143). Compared with uninfected WT plants, uninfected \textit{atg5}, \textit{atg7}, and \textit{atg18a} mutants had significantly higher basal JA levels and higher transcription levels of a JA-regulated defense gene, \textit{PDF1.2} (62, 143). Upon infection with the necrotrophic fungus \textit{Botrytis}, the \textit{PDF1.2} transcription level was greatly reduced in the \textit{atg} mutants compared with WT plants, despite the elevated basal level. This result suggests that autophagy negatively regulates JA-mediated \textit{PDF1.2} gene expression in healthy plants but positively regulates its level in infected plants (62).

SA was also elevated in uninfected \textit{atg} mutants (62, 68, 143). When \textit{atg} mutants were crossed with several SA-signaling defective mutants, both the early-senescence phenotype and the unrestricted PCD during \textit{Pst avrRpm1} infection were rescued (143). Crossing the \textit{atg} mutants to JA-related mutants did not produce the same result, suggesting a JA-independent mechanism (143). Upon treatment with the SA agonist benzo-(1,2,3)-thiadiazole-7-carbothioic acid, autophagy was induced in WT plants but not in the \textit{npr1} mutant (a
downstream component of SA signaling), indicating that SA-induced autophagy is dependent on NPR1 (143). Collectively, these results demonstrate that the premature senescence and the infection-induced spreading of HR-PCD are the result of an increased basal SA level in the \textit{atg} mutants, and that in WT plants autophagy functions to eliminate SA via a negative-feedback loop, which in turn suppresses the senescence and unrestricted PCD (141, 143).

The discovery of the involvement of SA signaling may help to explain three phenomena. First, the increased resistance to biotrophic pathogen infection in \textit{atg} mutants may result from the constitutively slightly enhanced SA level and therefore increased expression of SA-induced defense genes (67). Second, as mentioned above, the spreading of HR-PCD in \textit{atg} mutants has been observed only in the older leaves of older plants, and the increased resistance has been observed in younger leaves or younger plants (42, 94, 143). It is possible that in the younger plants, the \textit{atg} mutants have only a slightly increased SA level, which is responsible for the increased resistance during pathogen infection. However, the older \textit{atg} mutants generate more SA, which leads to the phenotypes of early senescence and hypersusceptibility to infection (38, 144). Autophagy can serve both a prodeath and a prosurvival role during biotrophic pathogen infection, and this determination is age dependent. Third, the role of autophagy during starvation stress is well understood: it helps to recycle nutrients, leading to increased sensitivity of \textit{atg} mutants to starvation stress. However, the role of autophagy in nutrient-rich conditions is still under investigation. It has long been known that \textit{atg} mutants have an early-senescence phenotype, but the underlying mechanism is unclear. Now it has been shown that senescence is modulated by the SA level, and autophagy functions to eliminate excessive SA, thus preventing premature senescence (140, 141).

1.1.7 Regulation of autophagy in plants

A milestone in our understanding of the regulation of autophagy was the identification of the TOR (target of rapamycin) kinase (13, 60). TOR is a PtdIns3K-related kinase that functions as a serine/threonine protein kinase and as a nutrient sensor that integrates multiple upstream
signals (45). The antibiotic rapamycin inhibits TOR kinase activity by forming a ternary complex with the FRB (FKBP12 and rapamycin binding) domain of TOR and the FKBP12 protein (15). In yeast, two TOR genes have been identified; plants, mammals, and other eukaryotes have only one TOR gene (95). In both yeast and animals, TOR forms two different functional complexes, TORC1 and TORC2, each of which contains distinct TOR binding partners (75). TORC2 controls spatial cell growth by regulating polarization of the actin cytoskeleton in a rapamycin-insensitive manner, whereas TORC1 is sensitive to rapamycin and controls temporal cell growth by promoting translation, transcription, ribosome biogenesis, and nutrient transport and negatively regulating autophagy (25, 75, 135). In yeast, TORC1 regulates the Atg1-Atg13-Atg17 complex depending on nutritional status. Under normal conditions, TORC1 is activated and causes the phosphorylation of Atg13; this hyperphosphorylated form of Atg13 has a low affinity for Atg1 and Atg17. Upon starvation or rapamycin treatment, TORC1 is inactivated and causes the dephosphorylation of Atg13; this hypophosphorylated form of Atg13 has a higher affinity for Atg1 and Atg17. The Atg1 kinase activity is activated after its interaction with Atg13 and Atg17, leading to the induction of autophagy (54, 135). Surprisingly, even though the key components of this regulatory pathway are conserved, in mammals and Drosophila the regulation and relationships between TORC1, Atg1, and Atg13 are divergent (40). Intriguingly, despite TOR being a key regulator for nutrient stress--induced autophagy, studies in HeLa cells and primary fibroblasts suggest that autophagy induced upon pathogen infection may be TOR independent (126).

The TOR homolog in Arabidopsis has been identified (83), but the investigation of TOR function is impeded for two reasons: first, disruption of the AtTOR gene is embryonic lethal (83), making the study of its postembryonic function difficult; and second, the Arabidopsis FKBP12 protein does not bind to rapamycin, making it insensitive to rapamycin, which means that this inhibitor cannot be used to study the TOR pathway in this species (77). Several strategies have been developed to overcome these problems. Transgenic Arabidopsis plants expressing yeast ScFKBP12 showed rapamycin sensitivity and displayed rapamycin-independent arrest of root growth (110). Arabidopsis transfer-DNA insertion lines with
Increased AtTOR expression and RNAi lines with decreased AtTOR expression showed that root and shoot growth are correlated with TOR expression level, indicating a role in growth regulation (23).

Recently, a mutant screen for suppressors of the root-hair cell-wall-formation mutant Irx1 led to identification of the rol5 mutant (66). ROL5 is structurally and functionally similar to the yeast Ncs6 protein, which is a component of the yeast TOR pathway. The authors suggested that the suppression of Irx1 by rol5 is based on alteration of TOR signaling, because treating Irx1 plants expressing yeast FKBP12 with rapamycin also relieves the Irx1 phenotype. This finding reveals that the TOR pathway is involved in cell wall formation in Arabidopsis (66).

Two studies provided direct evidence that TOR is a regulator of autophagy in photosynthetic species (72, 96). The green alga Chlamydomonas reinhardtii is sensitive to rapamycin, and autophagy was induced upon rapamycin treatment (96). In Arabidopsis, RNAi-AtTOR plants showed constitutive activation of autophagy (72). These results indicate that TOR function is conserved and that this factor serves as a negative regulator of autophagy in plants (Figure 1.5).

As mentioned above, TOR works in two different complexes. Although the TORC2 subunits remain to be identified in plants, some of the TORC1 binding partners have been identified (25). These include Raptor (35), which recruits substrates and presents them to TOR for phosphorylation, and LST8 (128), which stabilizes the TOR complex. In animal cells, rapamycin inhibits TOR activity partially by uncoupling the TOR/RAPTOR interaction (93). Two RAPTOR homologs have been identified in Arabidopsis: AtRAPTORIA and AtRAPTOR1B (4, 22). Both of them are expressed in growing tissues throughout the plant, although AtRAPTOR1B is the most highly expressed isoform (4, 22). Disruption of AtRAPTORIA has no obvious phenotype, whereas disruption of AtRAPTOR1B has been reported to have two contradictory phenotypes. One study showed that loss of AtRAPTOR1B causes seed abortion and a complete arrest of embryo development at a preglobular stage (22). Another showed that an Atraptor1B mutant is viable but has defects
in both root and shoot growth, resulting in delayed development (4). An *Atraptor1A Atraptor1B* double mutant exhibited normal embryonic development but was unable to maintain postembryonic meristem-driven growth (4). This phenotype discrepancy can potentially be explained by a variation in growth conditions, because the addition of 1% sucrose to the growth medium partially rescued this phenotype (4). Another TOR binding partner, LST8, has been identified in *Chlamydomonas reinhardtii* (24). CrTOR and CrLST8 exist in a large complex in which CrLST8 interacts with the CrTOR kinase domain; together, they colocalize to ER membranes. CrLST8 is able to complement the yeast lst8 mutant, implying a conserved function for this protein (24).

As a kinase, TOR signals through a combination of direct phosphorylation of downstream targets and repression of phosphatase activity (99). Recent research has shown that TOR controls embryogenesis and postembryonic development through its kinase domain in *Arabidopsis* (102). To date, several potential TOR substrates have been identified in plants. As mentioned above, the Atg1-Atg13-Atg17 complex is a TORC1 substrate, and in yeast and animals it is regulated by TORC1 depending on the nutritional status. In *Arabidopsis*, three putative ATG1 homologs and two putative ATG13 homologs have been identified (25); however, their functions and interactions have not been experimentally studied. In yeast, autophagy is regulated through PP2A (protein phosphatase type 2A), and a regulatory subunit of PP2A, Tap42/α4, has been shown to be a downstream substrate of the Tor protein. Tap42 is phosphorylated and tightly associates with PP2A under nutrient-rich conditions; upon starvation or rapamycin treatment, Tap42 is dephosphorylated and dissociates from PP2A (135). A plant homolog of Tap42/α4, Tap46, has been identified in both *Arabidopsis* and tobacco (*Nicotiana tabacum*) (2). Tap46 interacts with PP2A and is phosphorylated by TOR, suggesting that Tap46 is a direct substrate of TOR. Loss of Tap46 function results in growth arrest and activation of autophagy. These findings suggest that Tap46 functions as a component of the TOR signaling pathway (2).

Mei2 is a meiosis signaling molecule that has been suggested to be a potential TOR substrate in yeast (127). In *Arabidopsis*, the Mei2 homolog AML1 (*Arabidopsis* Mei2-like1) interacts
with RAPTOR1B in a yeast two-hybrid assay, which implicates AML1 as a candidate substrate of TOR (3). EBP1 (ErbB-3 epidermal growth factor receptor binding protein) is a nucleolar and cytoplasmic regulator of ribosome assembly and translation; it has been shown to regulate cell growth and proliferation in plants (44). In Arabidopsis, the expression of the EBP1 gene is correlated with TOR expression level, which indicates that EBP1 might be a possible substrate that functions downstream of TOR (23). S6K (ribosomal p70 S6 kinase) is a translational regulator that interacts with RAPTOR in Arabidopsis, which also makes it a potential TOR substrate (77). However, the relationship of these potential TOR substrates to autophagy induction has not been tested.

In animals, insulin is a key upstream regulator of the TOR pathway (7), and a maize insulin-related peptide, ZmIGF (Zea mays insulin-like growth factor), promotes cell growth via ZmTOR kinase activity (26). Additionally, maize has been shown to be responsive to rapamycin treatment (1) and the TOR-S6K pathway seems to be conserved (26), making maize a possibly superior model for studying the TOR-autophagy pathway in plants.

In yeast and animals, other signaling pathways that control autophagy have been identified. For example, the mammalian AMPK (AMP-activated protein kinase) and its yeast homolog SNF1 (sucrose nonfermenting 1) have been shown to regulate autophagy induction (135). In plants, SNF1/AMPK-related kinases (SNRKs) have been identified as SNF1/AMPK homologs. The Arabidopsis SNRK AKIN10 has been shown to induce several autophagy genes (8), suggesting a conserved role for AMPK in the regulation of autophagy in plants. However, further analysis is needed to confirm this interpretation.

### 1.1.8 Evidence for selective autophagy

In general, autophagy is considered to be a nonselective degradation process. However, in yeast and mammals, several studies have shown that certain organelles or protein aggregates can be selectively targeted by autophagy (for a more comprehensive review on selective autophagy, see 51, 103, 135). In yeast, the biosynthetic cytoplasm-to-vacuole targeting
pathway is a unique type of selective autophagy. The precursors of the vacuolar hydrolases Ape1 (aminopeptidase 1) and Ams1 (alpha-mannosidase 1) are delivered into the vacuole via double-membrane vesicles. The cargo receptor Atg19 recognizes and interacts with both the cargo and Atg8, and together they are transported to the PAS (135). Atg19 recognizes Atg8 through the AIM (see above) (88); this process also requires Atg11 as an adaptor. Various organelle-specific autophagy types have also been described in yeast (103)---for example, the selective degradation of mitochondria (mitophagy), peroxisomes (pexophagy), and ribosomes (ribophagy). In animals, two autophagic adaptors, p62/SQSTM1 (sequestosome 1) and NBR1, have been studied extensively and seem to play an important role during selective autophagy (51). Both p62 and NBR1 harbor a ubiquitin-associated domain that can bind ubiquitinated proteins and an AIM that can interact with Atg8/LC3. It has been proposed that both p62 and NBR1 are selective autophagy substrates and work as cargo receptors: first, protein aggregates are ubiquitinated, and then p62 and NBR1 are recruited to the ubiquitinated substrates; this is followed by interaction with Atg8, leading to the formation of autophagosomes around the cargo (51).

Plant NBR1 homologs were recently identified. In Arabidopsis, a single AtNBR1 (At4g24690) gene has been identified; it binds ubiquitinated proteins via a C-terminal ubiquitin-associated domain and interacts with AtATG8 through the AIM motif (116). In tobacco (Nicotiana tabacum), Joka2 has been identified as a structural and possibly a functional homolog of p62 and NBR1 proteins. Joka2 interacts with NtATG8f, and its expression level increases during nutrient deprivation conditions (146). These findings suggest that the selective autophagy machinery is conserved to some extent from yeast to animals and plants.

In plants, mitophagy and pexophagy have not been studied, although some research indicates that autophagy is capable of selectively removing certain proteins or structures. In BY-2 cells, a fusion protein between cytochrome b5 (Cyt b5) and red fluorescent protein (RFP) is transported to the vacuole for processing during nitrogen starvation (118). Interestingly, the vacuolar degradation rate of Cyt b5--RFP appears to be faster than that of other proteins, and
the percentage of colocalization between Cyt b5--RFP and yellow fluorescent protein (YFP)--Atg8 is higher than with mitochondria, indicating that the Cyt b5--RFP proteins are engulfed by autophagosomes at a higher frequency. This study suggests that there is some autophagy selectivity toward the Cyt b5--RFP proteins (118).

In *Arabidopsis*, RNS2 is a conserved ribonuclease of the RNase T2 gene family, and is essential for normal ribosomal RNA (rRNA) decay. *Arabidopsis* plants lacking RNS2 activity have longer-lived rRNA, accumulate RNA in the vacuole, and have constitutive autophagy (41). It has been proposed that RNS2 may participate in a possible “ribophagy”-like mechanism in plants, functioning in ribosome turnover under normal growth conditions. The absence of RNS2 disrupts cell homeostasis, resulting in constitutive autophagy to restore the housekeeping role of the ribophagy-like process (41, 76).

As mentioned above, plants recycle nutrients from senescing leaf chloroplasts to newly forming organs at least partially through the autophagy pathway. During leaf senescence, small vesicles containing only stromal components (RCBs) pinch off from the chloroplasts and are delivered to the vacuole. GFP-AtATG8 colocalizes with stroma-targeted DsRed in spherical bodies in the vacuole, and in an *atg5* mutant this vacuolar RCB accumulation is compromised. These data suggest that after the RCBs form, they are enclosed within autophagosomes and delivered to the vacuole for degradation (14, 49, 50, 124). The discovery of RCBs demonstrates that autophagy selectively degrades stromal fractions of chloroplasts during leaf senescence. This exciting finding indicates that autophagy can thereby degrade certain components of an organelle and not others.

Despite the emerging evidence for organelle-specific autophagy in plants, its underlying mechanism is still unclear. As mentioned above, cytoplasmic ubiquitinated proteins may be recognized by p62 and NBR1 and thus selectively incorporated into autophagosomes. However, how organelles or parts of organelles are recognized for degradation is unknown. In yeast, the mitophagy-specific protein Atg32 serves as a tag for mitochondrial degradation (56, 91). Atg32 localizes on the mitochondrial outer membrane, binds to the selective
autophagy adaptor Atg11, and along with mitochondria is further recruited to the vacuole for degradation. However, Atg32 and another mitophagy-specific protein, Atg33 (55), do not seem to have corresponding plant homologs (103). This raises the question of whether plants have other types of organelle-specific autophagy, such as mitophagy or pexophagy. If they do, do plants use a different set of genes or mechanisms? Future studies are anticipated to address these questions.

1.1.9 Summary points

1. The autophagy core machinery is conserved from yeast to animals and plants, although some of the identified yeast autophagy (ATG) genes are present as gene families in plants. Several autophagy-defective plants with impaired ATG genes have been characterized; most of them are able to complete their life cycles but display early senescence and hypersensitivity to starvation conditions.
2. Plant autophagy functions during various abiotic stresses, including nitrogen and carbon starvation as well as oxidative, salt, and osmotic stresses.
3. Plants have a basal level of housekeeping autophagy even under favorable growth conditions, which may function during vacuole biogenesis, help in the elimination of damaged proteins and organelles, and remobilize nutrients during leaf senescence and seed germination.
4. Plant autophagy is involved in PCD during pathogen infection. However, its exact role is unclear. The SA signal may mediate this process.
5. Just as in yeast and animals, evidence suggests that the TOR kinase serves as a negative regulator of autophagy in plants. Some components of the TOR complexes and TOR substrates have also been identified in plants.

1.1.10 Future issues

1. Although many yeast ATG gene homologs have been identified in plants, a few genes are missing from plant genomes, and some genes have expanded to gene families. It will be
interesting to determine whether novel autophagy genes exist in plants and whether the
genes within families have different roles under different circumstances.

2. The role of autophagy upon pathogen infection is still unclear. Several studies show that
autophagy can function in both a prosurvival and a prodeath role. It seems that both
pathogen type and plant age can affect the plant response, and the hormone SA is
implicated. The role of autophagy and the signals that determine autophagy function
during the immune response still need to be resolved.

3. Although TOR probably serves as an autophagy regulator in plants, its upstream signals
and downstream substrates that control the autophagy pathway still need to be investigated.
Also, TOR-independent regulatory pathways are yet to be unveiled.

4. Do plants also have organelle-specific autophagy? If so, do they use the same mechanisms
as in yeast?

5. Despite the important role of plant autophagy, autophagy-defective plants are able to
complete their life cycles. Whether a compensatory pathway exists that ensures survival
when the autophagy pathway is impaired remains an open question.

1.1.11 Mini-glossary

**ATG8-interacting motif (AIM):** a WXXL amino acid sequence that can be recognized by
ATG8.

**Autophagosome:** a double-membrane structure formed upon autophagy induction; it engulfs
portions of cytoplasm and delivers them to the vacuole for degradation.

**Hypersensitive response (HR):** a mechanism used by plants to limit the spread of pathogen
infection.

**PE:** Phosphatidylethanolamine (PE): a lipid component of biological membranes.

**RuBisCO-containing body (RCB):** a small vesicle containing only stromal components that
pinches off from the chloroplast during senescence.

**Salicylic acid (SA):** a plant hormone implicated in immune responses and senescence.

**TOR (target of rapamycin):** a PtdIns3K-related kinase that functions as a serine/threonine
protein kinase.
1.1.12 Acknowledgments

This work was supported by grants IOB-0515998 and MBC-1051818 from the National Science Foundation to D.C.B.

Disclosure statement

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

1.1.13 Literature cited


45. Hunter T. 1995. When is a lipid kinase not a lipid kinase? When it is a protein kinase. *Cell* 83:1–4


**49. Shows that stromal components released from chloroplasts in RCBs are packaged into autophagosomes and delivered to the vacuole for degradation by autophagy.**


**61. Shows that TE formation does indeed involve autophagy and identifies a Rab-type GTPase, RabG3b, that is required for this process.**


74. Provides the first report of functional analysis of autophagy during pathogen responses in plants.


76. Macintosh GC, Bassham DC. 2011. The connection between ribophagy, autophagy and ribosomal RNA decay. *Autophagy* 7:662--63


116. Reports on the analysis of NBR1, a potential selective autophagy receptor in plants; Reference 146 reports similarly on another possible receptor.


**134. Describes a role for autophagy during plant responses to oxidative stress.**


**142. Provides the first report of the use of ATG8 as an autophagosome marker in plants and demonstrates that *Arabidopsis* and yeast process ATG8s similarly.**


146. Reports on the analysis of Joka2, a potential selective autophagy receptor in plants; Reference 116 reports similarly on another potential receptor.
1.1.14 Figures and tables

**Figure 1.1. Pathway for autophagy in plant cells.** Upon induction of autophagy, a double-membrane structure called an autophagosome forms around a portion of the cytoplasm (cargo). The autophagosome then transports the cargo to the vacuole. During the fusion process, the outer autophagosome membrane fuses with the vacuole membrane, and the remaining single-membrane structure (termed an autphagic body) is delivered inside the vacuole. The autophagic bodies are then broken down by vacuolar hydrolases, and the products are exported from the vacuole to the cytoplasm for reuse.
Figure 1.2. Two ubiquitin-like conjugation systems in *Arabidopsis*. (Right) The AtATG8 conjugation system. The C-terminus of the AtATG8 proteins is cleaved by AtATG4; bound to an E1-like enzyme, AtATG7; transferred to an E2-like enzyme, AtATG3; and finally conjugated to phosphatidylethanolamine (PE). The orange Xs represent C-terminal sequences of AtATG8s that are cleaved by AtATG4s (142). There are eight members in the AtATG8 family; AtATG8h and AtATG8i do not possess additional amino acids after glycine and are not thought to be subject to cleavage by AtATG4. (Left) The AtATG12 conjugation system. AtATG12 proteins first bind to an E1-like enzyme, AtATG7; are then transferred to an E2-like enzyme, AtATG10; and are finally conjugated to AtATG5. The AtATG12-AtATG5 conjugate may also function as an E3-like enzyme during the AtATG8-PE conjugation. Whether AtATG12-AtATG5 interacts with AtATG16 to form a tetrameric ATG12-ATG5-ATG16 complex (as it does in yeast) and whether the ATG12-ATG5-ATG16 complex is also able to function as an E3-like enzyme are still unknown.
Figure 1.3. RNA interference (RNAi)--AtATG18a plants are hypersensitive to salt and drought stresses. Wild-type and RNAi-AtATG18a plants were grown in short-day conditions with regular watering every 2 days for 3 weeks, followed by 0.16-M salt or drought treatments for 5 weeks. In control conditions, little difference was observed between wild-type and RNAi-AtATG18a plants. However, under salt and drought stresses, the RNAi-AtATG18a plants showed decreased growth and survival. Figure adapted from Reference 74.
Figure 1.4. Autophagy is induced under salt and osmotic stresses. A fluorescence microscope was used to visualize autophagy induction in 7-day-old green fluorescent protein (GFP)--AtATG8e transgenic *Arabidopsis* roots. After treatment with 0.16-M NaCl or 0.35-M mannitol, numerous GFP-AtATG8e-labeled autophagosomes appeared, whereas few were present in control conditions. Arrows indicate GFP-labeled autophagosomes. Figure adapted from Reference 74.
Figure 1.5. Potential TOR signaling pathways in plants. The TORC1 complex, including TOR, RAPTOR, and LST8, senses and integrates multiple upstream signals such as nutrient starvation, insulin-like growth factors, or other stresses. TOR may serve as a negative regulator of autophagy. Some TORC1 substrates have been identified, including AML1, EBP1, Tap46 (a regulatory subunit of PP2A), and S6K. These substrates may function to control translation, cell growth, and possibly autophagy.
Table 1.1. Common phenotypes of *Arabidopsis* autophagy mutants and their corresponding affected genes.

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>Genes involved</th>
<th>References</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Accelerated senescence, hypersensitivity to starvation conditions, slower growth</td>
<td><em>ATG2, ATG4s, ATG5, ATG6, ATG7, ATG8s, ATG9, ATG10, ATG12s, ATG18a, VTI12</em></td>
<td>16, 27, 28, 34, 36, 94, 97, 113, 117, 132, 142, 143</td>
<td></td>
</tr>
<tr>
<td>Stunted growth, increased anthocyanin production, decreased silique production, abnormal pollen germination</td>
<td><em>ATG6</em></td>
<td>28, 36, 98</td>
<td></td>
</tr>
<tr>
<td>Hypersensitivity to drought and salt stresses</td>
<td><em>ATG18a</em></td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>Hypersensitivity to oxidative stress</td>
<td><em>ATG18a</em></td>
<td>107, 134</td>
<td></td>
</tr>
<tr>
<td>Altered resistance to pathogen infection</td>
<td><em>ATG2, ATG5, ATG6, ATG7, ATG9, ATG10, ATG18a</em></td>
<td>42, 62, 68, 94, 125, 143</td>
<td></td>
</tr>
</tbody>
</table>
1.2 Dissertation organization

This dissertation summarizes my research on the function and the regulatory pathways of autophagy in *Arabidopsis thaliana*.

Chapter 2 demonstrates a role for autophagy in the response to high saline and osmotic stresses in Arabidopsis. My results suggested that autophagy is induced by these stresses and autophagy-defective RNAi-AtATG18a plants are more sensitive to these stresses than wild-type plants. We have further shown that autophagy can be activated by NADPH oxidase-dependent or -independent pathways.

Chapter 3 shows that the Target of Rapamycin (TOR) gene, which was previously shown to be a negative regulator in both yeast and animal systems, also negatively regulates autophagy in Arabidopsis. RNAi-AtTOR transgenic lines with decreased AtTOR transcript level were generated. These RNAi lines were demonstrated to have constitutive autophagy even under favorable growth conditions.

Chapter 4 presents a role for autophagy in the morphological changes in ER structure during ER stress in Arabidopsis. Both confocal and electron microscopy analysis showed that portions of the ER are delivered to the vacuole by autophagy during ER stress. We further demonstrate that an ER stress sensor, AtIRE1b, is required for ER stress-induced autophagy. However, the IRE1b splicing target, bZIP60, does not seem to be involved, suggesting that a novel signaling pathway regulates ER stress-induced autophagy in plants.

Chapter 5 summarizes the conclusions of this research and discusses areas of future work.
CHAPTER 2
AUTOPHAGY IS REQUIRED FOR TOLERANCE OF DROUGHT AND SALT STRESS IN PLANTS

A paper published in Autophagy*
Yimo Liu¹,²†, Yan Xiong²,³† and Diane C. Bassham¹,²‡

1. Interdepartmental Genetics Program
2. Department of Genetics, Development and Cell Biology, 253 Bessey Hall, Iowa State University, Ames, Iowa, 50011
3. Current address: Department of Molecular Biology, Massachusetts General Hospital, Department of Genetics, Harvard Medical School, Boston, Massachusetts 02114, USA

2.1 Abstract

Autophagy is a protein degradation process in which cells recycle cytoplasmic contents when subjected to environmental stress conditions or during certain stages of development. Upon the induction of autophagy, a double membrane autophagosome forms around cytoplasmic components and delivers them to the vacuole or lysosome for degradation. In plants, autophagy has been shown previously to be induced during abiotic stresses including nutrient starvation and oxidative stress. In this paper, we demonstrate the induction of autophagy in high salt and osmotic stress conditions, concomitant with the upregulation of expression of an Arabidopsis thaliana autophagy-related gene AtATG18a. Autophagy-defective RNAi-AtATG18a plants are more sensitive to salt and drought conditions than wild-type plants, demonstrating a role for autophagy in the response to these stresses. NADPH oxidase inhibitors block autophagy induction upon nutrient starvation and salt stress, but not during

† These authors contributed equally to this work. Y.X. generated the AtATG18apro: GUS transgenic plants. Experiments described in Figure 2.2 (A) (C), 2.3 (A), 2.4 (A), S2.3 were conducted by Y.X. All the other experiments described in this chapter were conducted by Y.L.
‡ Author for correspondence
osmotic stress, indicating that autophagy can be activated by NADPH oxidase-dependent or -independent pathways. Together our results indicate that diverse environmental stresses can induce autophagy and that autophagy is regulated by distinct signaling pathways in different conditions.

**Key words**: Autophagy, AtATG18, salt stress, osmotic stress, NADPH oxidase, drought, Arabidopsis

**Abbreviations**
DPI, diphenylene iodonium; MDC, monodansylcadaverine; MV, methyl viologen; ROS, reactive oxygen species; WT, wild type

### 2.2 Introduction

Plants, in common with other eukaryotes, have sophisticated mechanisms to degrade and recycle unwanted or damaged intracellular components during development or under certain stresses.\(^1\) While the ubiquitin-proteasomal system is in general used for the degradation of short-lived proteins, autophagy degrades and recycles long-lived proteins and organelles.\(^3\)

Autophagy, which means “self-eating”,\(^4\) is a protein degradation process in which cells recycle cytoplasmic contents when subjected to environmental stress conditions or during certain stages of development. Macroautophagy involves the formation of cytoplasmic autophagosomes that enclose portions of the cytosol. Upon the induction of autophagy, a double membrane autophagosome forms around cytoplasmic components and delivers them to the vacuole or lysosome for further degradation. In this paper, we will focus on macroautophagy, herein referred to as autophagy.

Although autophagy was described in plants a number of years ago,\(^5\) more recently the molecular mechanism and regulatory pathways have begun to be studied. Our understanding of autophagy has been greatly enhanced by mutagenesis studies in yeast,\(^8\) by which many autophagy–related (ATG) genes were identified. These genes can be divided into several major functional groups,\(^11\) including an Atg9 cycling system,\(^12\) a phosphatidylinositol 3-OH kinase (PI(3)K) complex\(^14\) and two ubiquitin-like (Ubl) conjugation systems.\(^16\)
These studies in yeast have been useful in identifying plant homologues of genes required for autophagy and in providing direction for investigating their molecular function. The AtATG8-PE/AtATG12-AtATG5 conjugates and their associated enzymes are the most extensively studied plant autophagy components and were found to be essential for plant autophagy.\textsuperscript{16-22} Disruption of additional Arabidopsis thaliana ATG genes, including \textit{AtATG2}, \textit{AtATG6}, \textit{AtATG9} and \textit{AtATG18}, also caused autophagy defects.\textsuperscript{12, 23-25} Autophagy-defective mutants showed no morphological differences from wild type plants during most of their life cycle; however, leaf senescence and inflorescence bolting was accelerated in these mutants, and this phenotype was more severe when grown in either carbon- or nitrogen-deprivation conditions.\textsuperscript{12, 21, 24, 26} Autophagy is essential for the degradation of oxidized proteins during oxidative stress in plants,\textsuperscript{27, 28} in degrading aggregated proteins in nutrient-starved plant cells,\textsuperscript{29} and in limiting the cell death response during pathogen attack.\textsuperscript{25, 30}

High salinity and drought stress are two of the most common environmental stresses encountered by plants,\textsuperscript{31-33} and have some common features but are regulated differently. High salinity differs from drought stress in creating ionic stress in addition to osmotic stress,\textsuperscript{34} including Na\textsuperscript{+} toxicity and K\textsuperscript{+} deficiency; osmotic stress can cause membrane disruption and enzyme dysfunction.\textsuperscript{35} At the same time, both stresses can cause oxidative damage to the cell, probably increasing the production of reactive oxygen species (ROS) and damaged proteins.\textsuperscript{36} Plants have developed several mechanisms to tolerate these stresses, such as controlling the growth rate by altering cell wall biosynthesis, protein synthesis and cell division.\textsuperscript{37} Many genes have been shown to respond to these stresses and may be involved in mechanisms of stress tolerance,\textsuperscript{34} for example, the salt-overly-sensitive (SOS) genes regulate the vacuolar Na\textsuperscript{+}/H\textsuperscript{+} transporter system under salt stress.\textsuperscript{38, 39} Autophagy degrades oxidized proteins during oxidative stress,\textsuperscript{27} but the potential role of autophagy in the response to salt and osmotic stresses is still unknown. It has been shown that autophagy-related proteins function in plant responses to salt and osmotic stress,\textsuperscript{28, 40} although the relationship to autophagy is not clear.
In this paper, we show that autophagy is induced by high salinity and osmotic stresses, and that autophagy-defective plants are more sensitive to these conditions. We also demonstrate that autophagy is regulated by distinct signaling pathways under different stress conditions.

### 2.3 Results

**Expression pattern of AtATG18a under different stress conditions**

We have shown previously that AtATG18a is essential for autophagy induction under starvation and oxidative stress conditions and during senescence\(^{24,27}\) and that its expression is up-regulated under these conditions. To extend the analysis of conditions in which AtATG18a may function, expression during salt stress induced by 0.16M NaCl and osmotic stress induced by 0.35M mannitol was determined by semi-quantitative RT-PCR. To confirm that these conditions are effective in causing salt and osmotic stresses, the expression of salt and osmotic stress-induced genes was also analyzed. SOS1 gene was selected as a salt stress-specific marker since its transcript level increases under salt stress but not osmotic stress.\(^{41}\) DREB2A and RD29A were selected as osmotic stress markers since their transcript levels increase under both drought and salt stresses.\(^{42}\) Seven-day-old WT seedlings grown in control MS plates were transferred to MS medium, MS+0.16M NaCl plates and MS+0.35M mannitol plates. Seedlings were collected at 0, 1, 2, 4 and 8h followed by RNA extraction and RT-PCR using gene-specific primers (Table 2.1; Figure 2.1A). The AtATG18a gene was expressed at a basal level in the control condition, and its transcript level increased during both salt and osmotic stress. As expected, the marker genes DREB2A, RD29A and SOS1 all had an increased transcript level during salt stress, whereas DREB2A and RD29A transcripts were increased during osmotic stress. We conclude from these results that expression of the AtATG18a gene is up-regulated by both salt and osmotic stresses.

To analyze in more detail the cell types in which AtATG18a is expressed, a 1kb fragment upstream of the start codon corresponding to the AtATG18a promoter was fused with the GUS (β-glucuronidase) gene and AtATG18apro:GUS transgenic plants were generated. Two independent transgenic lines were used in all later experiments and behaved in the same way.
Homoygous transgenic *AtATG18apro: GUS* seeds were germinated on standard MS (Murashige–Skoog) medium plates and grown for one week. The seedlings were then transferred to control MS plates, MS plates containing 10mM MV, 0.16M NaCl or 0.35M mannitol or MS plates lacking sucrose (grown in the dark) or nitrogen for 4 days. The seedlings were collected and submerged in GUS staining solution for 16h, destained in 70% ethanol for 16h and observed with a light microscope (Figure 2.1B-G). For all conditions, the cotyledons and leaves showed weak and somewhat variable staining, except for strong staining in hydathodes (data not shown). The reason for this staining pattern is not clear, but a number of genes are expressed in the hydathodes for unknown reasons. As staining in aerial tissues was variable, and as our assays for autophagy typically use root tissue, we focused on the staining pattern in roots. In *AtATG18apro: GUS* plants under control conditions the roots have no visible GUS activity (Figure 2.1B) while during nutrient starvation (sucrose or nitrogen; Figure 2.1C, 2.1D) and oxidative stress (+MV; Figure 2.1E) roots were evenly stained, indicating increased *AtATG18a* expression level. Interestingly, the staining pattern in the +NaCl and +mannitol conditions was different from the nutrient starvation and oxidative stresses. After 6h incubation on 0.16M NaCl or 0.35M mannitol plates, the roots stained evenly as for the oxidative stress and starvation conditions (see Figure 2.4C). However, after 4 days incubation under these conditions (Figure 2.1F, 2.1G), only the lateral root branch points and the root tips were stained. Other parts of the roots were either weakly stained or no staining was visible at all. One possibility is that the lateral root branch point is where epidermal cells separate and the salt or mannitol creates severe ionic and osmotic stresses within the root, leading to increased *AtATG18a* expression. Some autophagy genes are known to increase in expression only transiently upon exposure to stress conditions and then decrease back to basal levels. After an immediate increase in expression upon initial exposure to salt or mannitol, *AtATG18a* expression may decrease again in the majority of the root tissue by 4 days incubation time.

**Autophagy is induced by salt and osmotic stresses**

As shown in Figure 2.1B-G, *AtATG18a* expression level is increased during salt and osmotic stresses. Since in other conditions up-regulation of *AtATG18a* expression correlates with the
induction of autophagy, the possible induction of autophagy under salt and osmotic stresses was investigated. Wild type (WT) seeds or transgenic seeds expressing the autophagosome marker GFP-ATG8e were first germinated on control MS plates and grown for one week. The one-week-old seedlings were transferred to control MS plates or to MS plates containing 0.16M NaCl or 0.35M mannitol for 6-8h to cause salt and osmotic stresses respectively. Autophagosomes were detected by fluorescence microscopy after staining WT seedlings with the autophagosome-specific dye monodansylcadaverine (MDC) or by direct visualization of the GFP-ATG8e marker (Figure 2.2A and C). We have shown previously that MDC labeling and GFP-ATG8e co-localize on autophagosomes in Arabidopsis upon induction of autophagy. Numerous autophagosomes were observed in WT and GFP-ATG8e plants under both salinity and osmotic stresses, while in control conditions autophagosomes were rarely seen. The number of MDC-stained autophagosomes per root section was also analyzed (Figure 2.2B). Compared to control conditions, significantly more autophagosomes were detected upon salt and mannitol treatments (P-value=0.000040 for mannitol treatment, P-value=0.00017 for salt treatment). These data indicate that salt and osmotic stresses can induce autophagy.

AtATG18a is required for autophagy induced by starvation and oxidative stress, and its expression is induced by salt and mannitol; we thus hypothesized that AtATG18a may also be required for salt and mannitol-induced autophagy. RNAi-AtATG18a transgenic plants have reduced AtATG18a transcription level and are defective in autophagosome formation. One week old RNAi-AtATG18a seedlings were transferred to control MS plates or MS plates containing 0.16M NaCl or 0.35M mannitol for 6-8h, followed by MDC staining (Figure 2.2A). In these transgenic lines the number of autophagosomes was dramatically reduced under both salt and mannitol treatment conditions, indicating that salt or mannitol-induced autophagy is dependent on the AtATG18a gene.

RNAi-AtATG18a plants are more sensitive to salt and osmotic treatment

Figure 2.2A indicates that AtATG18a is not only essential for starvation- and oxidative stress-induced autophagy, but also for salt- and osmotic- stress induced autophagy. To
determine whether \textit{AtATG18a} is required for plant tolerance of salt and osmotic stresses, the phenotypes of RNAi-\textit{AtATG18a} and WT plants were assessed in these conditions. One-week-old seedlings grown on MS solid medium were transferred to medium with 0.16M NaCl or 0.35M mannitol for 10 days. After NaCl treatment, the growth of both WT and RNAi-\textit{AtATG18a} plants was inhibited, but the RNAi-\textit{AtATG18a} plants were more sensitive to these conditions. After 10 days of salt treatment, RNAi-\textit{AtATG18a} plants were chlorotic, whereas wild-type seedlings were still green (Figure 2.3A). To confirm this phenotype, chlorophyll was extracted from WT and RNAi-\textit{AtATG18a} seedlings at 0, 5 and 10 days and quantified by spectrophotometry (Figure 2.3B). The chlorophyll content decreased more rapidly under salt stress in the RNAi-\textit{AtATG18a} plants than the WT plants (5d P value=0.0175, 10d P value=0.0472).

Mannitol treatment also inhibited the growth of both wild-type and RNAi-\textit{AtATG18a} plants. However, the growth of RNAi-\textit{AtATG18a} seedlings was inhibited more strongly, and RNAi-\textit{AtATG18a} leaves contained increased anthocyanin levels (Figure 2.3A), indicating increased sensitivity to the osmotic stress. Anthocyanin content was determined for WT and RNAi-\textit{AtATG18a} seedlings after 0, 5 and 10 days of osmotic stress treatment (Figure 2.3B); the anthocyanin level increased more rapidly in the RNAi-\textit{AtATG18a} plants than the WT plants (5d P value=0.0143, 10d P value=0.0207). These results indicate that the RNAi-\textit{AtATG18a} plants are more sensitive to salt and osmotic conditions, and therefore \textit{AtATG18a} may function in the response of plants to these stresses.

To determine whether autophagy also functions in salt and osmotic stress (drought) tolerance when plants are grown in soil, RNAi-\textit{AtATG18a} and WT seeds were germinated and grown in short-day conditions with regular watering every 2 days for 3 weeks, at which time salt and drought treatments were initiated. For salt stress, the 3-week-old seedlings were watered with 0.16M NaCl solution every 2 days; for drought stress, water was withheld. In control conditions, watering was continued every 2 days throughout the experiment. After 5 weeks of drought or salt treatment, the phenotypes were assessed (Figure 2.3C). In control conditions, no significant differences were observed between WT and RNAi-\textit{AtATG18a}
plants. The drought and salt treatment affected the growth of both WT and RNAi-AtATG18a plants to some extent, but this effect were much more severe in the RNAi-AtATG18a plants. Upon drought treatment, WT leaves were still green and turgid; however, the RNAi-AtATG18a plants were desiccated by this time. Upon salt treatment, both the WT and RNAi-AtATG18a plants were smaller than in control conditions, but growth was retarded much more severely in the RNAi-ATATG18a plants. These observations suggest that autophagy is necessary for plants to tolerate high salt and drought conditions.

Some evidence suggests that autophagy may be involved in the degradation of storage reserves during seed germination, but autophagy mutants have no obvious defects in germination rate or extent. The effect of disrupting autophagy on seed germination in salt or osmotic stress was analyzed. After surface sterilization and cold treatment in the dark for 2 days, WT and RNAi-AtATG18a seeds were placed on control plates or plates containing 0.16M NaCl or 0.35M mannitol. The numbers of germinating seeds was counted every two days (Figure 2.3D). In control conditions, the WT and RNAi seeds behaved the same way; more than 98% of the seeds had germinated after 2 days. In mannitol stress, the germination of both WT and RNAi seeds was delayed compared to the control. A small difference was seen between WT and RNAi seeds after 6 days, with the RNAi seeds showing slightly delayed germination compared with the WT seeds, but this was only marginally significant (P value= 0.045). Under NaCl stress, germination was delayed still further for both WT and RNAi seeds. Germination of the RNAi seeds lagged behind that of WT seeds, with statistically significant differences seen after 4 days (34.5±5.2% germination for WT compared with 12.4±9.2% for RNAi; P value=0.0029), suggesting that autophagy may be involved in salt tolerance during seed germination.

**Autophagy is regulated by distinct pathways during different abiotic stresses.**

Autophagy is known to be induced by nutrient starvation and senescence. Under these conditions, and during salt and osmotic stress conditions, one common characteristic is the production of reactive oxygen species (ROS), which can act as signal molecules to activate stress response and defense pathways. We therefore hypothesized that ROS may function
as signal molecules to induce autophagy in abiotic stresses. A major pathway for production of signaling ROS under various abiotic and biotic stresses is plasma membrane NADPH-dependent oxidase, which can be inhibited by the chemical inhibitors diphenylene iodonium (DPI) and imidazole. The general antioxidant ascorbic acid can also remove ROS. To study the potential role of NADPH oxidase and ROS in autophagy, 7-day-old seedlings were transferred to stress conditions and then treated with DPI, imidazole or ascorbic acid. For starvation conditions, 7-day-old WT or GFP-AtATG8e transgenic plants seedlings were transferred to medium lacking sucrose or nitrogen for 4 days, then transferred to liquid MS medium with 20μM DPI or an equivalent volume of solvent (DMSO) as control, 20mM imidazole or 2mM ascorbic acid for 4h. For salt and osmotic stress treatment, 7-day-old WT or GFP-AtATG8e transgenic plants seedlings were transferred to liquid MS medium plus 0.16M NaCl or plus 0.35M mannitol with 20μM DPI or equivalent DMSO, 20mM imidazole or 2mM ascorbic acid for 4h. To observe autophagosomes, WT seedlings were stained with MDC (Figure 2.4A, S2.1A, and S2.2A); the autophagosomes in GFP-AtATG8 transgenic plant were visualized via the GFP label (Figure S2.1B, S2.2B).

Under control conditions, autophagosomes were rarely seen, irrespective of the presence of inhibitors. This indicates that neither DPI, imidazole nor ascorbic acid induce autophagy. Under the stress conditions (-nitrogen,-sucrose, +mannitol,+NaCl), numerous autophagosomes were observed in the absence of DPI, imidazole or ascorbic acid treatment, indicating the induction of autophagy. However, after adding DPI (Figure 2.4A), imidazole (Figure S2.1A, 2.1B) or ascorbic acid (Figure S2.2A, 2.2B), no punctate structures were observed in starvation and salt stress conditions, but they were still present after mannitol treatment. This suggests that the induction of autophagy is inhibited by these treatments in nutrient and salt stress, but is not inhibited during osmotic stress. To quantitate the effect of NADPH oxidase inhibition on autophagosome formation, the number of autophagosomes per root section was analyzed after imidazole treatment (Figure 2.4B). This confirmed that for starvation and salt stresses, the autophagosomes number dramatically decreased upon incubation with imidazole, whereas imidazole had no effect on autophagosome number in mannitol treatment. These results suggest that autophagy is differentially regulated under
starvation, salt and osmotic stresses: in nutrient starvation and salt stress, autophagy is most likely regulated by an NADPH oxidase-dependent pathway, involving ROS; whereas in osmotic stress, autophagy is regulated by an NADPH oxidase-independent pathway, which may not involve ROS as a signal.

To determine whether NADPH oxidase signaling acts upstream or downstream of transcriptional activation of the AtATG18a gene, 7-day-old AtATG18ap: GUS transgenic seedlings were transferred to stress conditions and then treated with imidazole. For starvation conditions, seedlings were transferred to medium lacking sucrose or nitrogen for 4 days then transferred to liquid MS medium supplemented with or without 20mM imidazole for 6h. For the salt and osmotic stress conditions, seedlings were transferred to liquid MS medium plus 0.16M NaCl or plus 0.35mM mannitol plus or minus 20mM imidazole for 6h. Longer incubation times were avoided due to potential toxicity or other indirect effects of the inhibitor. GUS activity was assayed and observed with a light microscope. As shown in Figure 2.4C, imidazole treatment inhibits AtATG18a induction in starvation and +NaCl conditions, but does not prevent its expression increase in +mannitol conditions. These observations further confirm that autophagy can be regulated by both NADPH oxidase dependant and independent pathways, and suggest that NADPH oxidase acts upstream of upregulation of AtATG18a expression.

2.4 Discussion

Autophagy is a non-specific protein degradation pathway induced in multiple environmental stress conditions and certain stages of development in plants, such as nutrient starvation, oxidative stress and senescence. In this paper, we demonstrate a role for autophagy in the response to high salinity and osmotic/drought stresses. First, the mRNA level of the autophagy-related gene AtATG18a was found to increase during salt and osmotic stresses (Figure 2.1). Second, autophagy was found to be induced under these conditions, as measured by MDC staining and recruitment of GFP-AtATG8 to autophagosomes (Figure 2.2). Third, the growth of RNAi-AtATG18a plants was more sensitive to salt and
osmotic/drought conditions. Growth was retarded compared with WT plants both on plates (Figure 2.3A) and in soil (Figure 2.3C) and seed germination was also delayed under salt treatment (Figure 2.3D). Together, this evidence suggests that autophagy is involved in plant survival during exposure to salt and drought environments.

The precise function of autophagy during salt or osmotic stress has yet to be determined. Under oxidative stress, autophagy is responsible for degrading oxidized proteins, which otherwise cause toxicity, and oxidized proteins accumulate substantially in autophagy-defective plants under these conditions when compared to WT plants. Salt and osmotic stresses can also increase the production of ROS and cause damage to proteins; one possibility is that autophagy might be responsible for degrading oxidized proteins under salt and osmotic stresses. However, oxidized protein level was analyzed in both WT and RNAi-AtATG18a plants by derivatization of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) and detection with 2,4-dinitrophenol (DNP) antibodies. While the oxidized protein level increased somewhat over time in these stresses in both WT and RNAi-AtATG18a plants, no significant difference was observed between genotypes (Figure S2.3), indicating that degradation of oxidized proteins is unlikely to be a major function of autophagy under these conditions. Autophagy has also been shown to transport aggregated proteins to the plant vacuole for degradation, and damage to proteins during salt or osmotic stress may cause aggregation. However, we were unable to demonstrate a significant difference in the level of SDS-resistant protein aggregates by SDS-PAGE between WT and RNAi-AtATG18a plants (data not shown). While we cannot rule out an effect on aggregated protein that is not detectable by this procedure, these results indicate that autophagy may act in some as yet unidentified role during these stresses.

In addition to their role in host defense during infection, ROS function as signal molecules during biotic and abiotic stresses in plants as well as other organisms. During starvation in animal cells, autophagy is regulated via superoxide production and in yeast, ROS directly regulate the Atg4 protein during starvation, thus activating autophagy. Recently, a role for the NOX2 NADPH oxidase in autophagy activation was demonstrated in animal
cells during the immune response to bacterial pathogens, with ROS being required for recruitment of the ATG8 homolog LC3 to phagosomes. In Arabidopsis, autophagy was found to be regulated by different pathways under different abiotic stresses: starvation- and salt stress-induced autophagy is dependent on NADPH oxidase activity, while osmotic stress-induced autophagy is not. The specific NADPH oxidase isoform(s) responsible for autophagy induction is not yet known. Arabidopsis contains 10 genes encoding NADPH oxidase homologs (AtrbohA - AtrbohJ), complicating genetic analysis. We analyzed autophagy induction in the previously characterized NADPH oxidase mutants (Atrbohc, d and f), but no difference was seen compared with WT plants (data not shown). It is likely that either an NADPH oxidase other than those tested is involved in autophagy induction, or that functional redundancy exists between genes, and a combination of multiple knockouts would be required to observe an effect.

We hypothesize that the initial pathways for detection of the salt and osmotic stresses are distinct, and converge at or before the initiation of autophagosome formation. It is not yet known whether these pathways involve known salt or osmotic stress-responsive genes, or novel pathways and components. In plants, there are two major pathways for osmotic stress response, an ABA-dependent and an ABA-independent but DREB2-dependent pathway. As direct addition of ABA to plants fails to induce autophagy, and ABA biosynthetic mutants have no defect in autophagy induction (data not shown), it is possible an ABA-independent pathway may play a role in autophagy induction during osmotic stress.

Upon analysis of autophagy induction by multiple stress conditions, evidence suggests that AtATG18a expression pattern reflects the activation of autophagy in Arabidopsis. Previously, AtATG18a was shown to be up-regulated under starvation and osmotic stress conditions, and here we show increase in expression under salt and osmotic stress, all of which are able to induce autophagy. In addition, imidazole treatment inhibits this increase in AtATG18a expression, concomitant with inhibition of autophagy. This suggests that the expression of AtATG18a can be used as a marker to predict the activity of the autophagy pathway. With the availability of numerous microarray databases analyzing gene expression under many
different conditions, the AtATG18a expression pattern has the potential to predict whether autophagy can be induced at different developmental stages or under different conditions, and may thus open up areas for future study of the physiological functions of autophagy in plants.

### 2.5 Methods

**Plant materials and growth conditions**

For phenotypic analysis in soil under salt and drought conditions, *Arabidopsis thaliana* plants were grown under short-day conditions (8h light) at 22°C. Three weeks after germination plants were either watered with 0.16 M NaCl solution (salt treatment) every 2 days or water was withheld (drought treatment), and growth continued for an additional 5 weeks. In control conditions, watering was continued every 2 days throughout the experiment.

For plate assays, *Arabidopsis thaliana* seeds were surface sterilized in 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. Arabidopsis plants were grown under long-day conditions (16h light) at 22°C on nutrient solid MS medium [Murashige–Skoog Vitamin and Salt mixture (Caisson, Cat. No. MSPA0910), 1% (w/v) sucrose, 2.4 mM MES (pH 5.7) and 0.8% (w/v) phytagar].

For methyl viologen (MV), mannitol and salt treatment, 7-day-old seedlings grown on nutrient solid MS medium were transferred to the same medium containing 10 mM MV, 0.35M mannitol, or 0.16M NaCl and incubated for the indicated times. For sucrose starvation experiments, 7-day-old seedlings grown on nutrient solid MS medium were transferred to the same medium without sucrose and incubated in the dark. For nitrogen starvation experiments, 7-day-old seedlings grown on nutrient solid MS medium were transferred to nitrogen-depleted MS solid medium as described and incubated for the indicated times. For controls, 7-day-old seedlings were transferred to fresh control MS medium.
RT-PCR analysis of AtATG18a, DREB2A, RD29A and SOS1 expression

Total RNA was extracted from each sample using the TRIzol reagent (Invitrogen, Cat. No. 15596026) followed by DNase I treatment (Invitrogen, Cat. No. 18068015). For the reverse transcription, cDNAs were generated using Superscript III reverse transcriptase (Invitrogen, Cat. No. 18080044) and an oligo dT primer. Primers used for PCR are shown in Table 2.1.

Generation of AtATG18apro: GUS transgenic plants

An AtATG18a (At3g62770) 1kb promoter fragment was synthesized by PCR from total genomic DNA using gene-specific primers 5’-HindIII 5’-AAGCTTTTGATTGTCAACGTCTGGAAGCCT-3’ and 3’-XbaI 5’-TCTAGAGGAAGAATGATTTGAAGGAATC-3’. The PCR product was sequenced for verification and ligated into the pGPTV-BAR vector upstream of the GUS gene. This construct was introduced into Agrobacterium tumefaciens strain GV3101 by electroporation, and then into Arabidopsis Col-0 plants by Agrobacterium-mediated transformation using the floral dipping method. Transgenic plants were identified using BAR resistance. BAR is the gene which gives resistance to the herbicide glufosinate or bialaphos. Homozygous T2 transformant seeds were used for further studies.

GUS staining

Seedlings grown under the indicated conditions were collected and submerged for 16h in the following staining solution: Triton/ethanol stock (Triton X-100: ethanol: water; 1:4:5), 0.5M KPO₄ buffer (pH 7.0), 0.1M ferricyanide solution (pH 7.0), 0.1M ferrocyanide solution (pH 7.0), 10 mg/ml bromo-4-chloro-3-indolyl-β-D-glucopyranoside in dimethyl sulphoxide (5:470:2:2:25), 1% (v/v) triton X-100/ethanol solution, 94% (v/v) KPO₄ buffer (0.5M, pH 7.0), 0.4% (v/v) ferricyanide solution (0.1M pH 7.0), 0.4% (v/v) ferrocyanide solution (0.1M pH 7.0), 0.05% (w/v) 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside. Plants were washed once with 70% (v/v) ethanol, destained in 70% ethanol for 16h and observed by light microscopy.
MDC staining and microscopy

GFP-AtATG8e transgenic plants were generated previously and observed using a FITC (fluorescein isothiocyanate)–specific filter. Wild-type and RNAi-AtATG18a seedlings were stained with MDC as previously described. After staining, the roots of seedlings were observed using fluorescence microscopy with a DAPI (4',6-diamidino-2-phenylindole)–specific filter. For counting of autophagosomes, pictures were taken of equivalent regions of each root for multiple seedlings in GFP-AtATG8e plants or after MDC staining. The number of autophagosomes visible in each image was counted for each treatment. In all cases, unpaired t-tests were performed to determine statistical significance, defined as P-value < 0.05.

Determination of chlorophyll and anthocyanin content

Chlorophyll was extracted in 80% acetone and absorbance measured at 663 and 646 nm for calculation of total chlorophyll content per gram fresh weight. For anthocyanin content, seedlings were homogenized in propanol: HCl: water (18:1:81) and further extracted in a boiling water bath for 3 min. The absorbance of the supernatant was measured at 535 and 650 nm and total anthocyanin content per gram fresh weight determined.

Seed germination

After surface sterilizing and cold treatment, Arabidopsis wild type and RNAi-AtATG18a seeds were grown on solid MS medium or the same medium containing 0.35M mannitol or 0.16M NaCl. The number of germinated seeds was counted after 2, 4, 6 and 8 days, defined as seeds with a visibly protruding radicle. At least 100 seeds were used for each genotype and each treatment, and three independent replicates were performed.

Inhibitor treatments

For nitrogen starvation, 7-day-old seedlings grown on nutrient solid MS medium were transferred to nitrogen-depleted MS solid medium and incubated for 4 days, then transferred to MS liquid medium plus or minus 20μM DPI, 20mM imidazole or 2mM ascorbic acid for 4-6 h.
For mannitol and NaCl treatment, 7-day-old seedlings grown on nutrient solid MS medium were transferred to the MS liquid medium containing 0.35M mannitol or 0.16mM NaCl plus or minus 20μM DPI, 20mM imidazole or 2mM ascorbic acid for 4-6 h. The solvent for DPI was dimethyl sulfoxide (DMSO); an equivalent volume of DMSO was added to controls.

2.6 Acknowledgements

We thank Randall Den Adel and Tracey M. Pepper for assistance with the microscopy and Gehyun Lee and Hansaa Abbasi for assistance with the seed germination experiments. This work was supported by grant no. IOB-0515998 from the National Science Foundation to DCB.

2.7 References


2.8 Figures and tables

**Figure 2.1. Expression pattern of *AtATG18a* under different conditions.** (A) Seven-day-old WT seedlings grown on control MS plates were transferred to MS, MS+0.16M NaCl plates or MS+0.35M mannitol plates. Seedlings were collected at the indicated times followed by RNA extraction and RT-PCR. *DREB2A* and *RD29A* were used as osmotic stress controls; *SOS1* was used as a salt stress control; 18S RNA was a loading control. (B-G) Homozygous transgenic *AtATG18apro: GUS* seeds were germinated on standard MS medium plates and grown for one week. The seedlings were then transferred to control MS plates (B), MS plates lacking sucrose (grown in the dark) (C) or lacking nitrogen (D), or MS plates containing 1mM MV (E), 0.16M NaCl (F) or 0.35M mannitol (G) for 4 days. The seedlings were collected and submerged in GUS staining solution for 16h, destained in 70% ethanol for 16h and observed with a light microscope.
Figure 2.2. Autophagy is induced by salt and osmotic stresses. (A) One week old WT or RNAi-AtATG18a seedlings were transferred to control MS plates or to MS plates containing 0.16M NaCl or 0.35M mannitol for 6-8 h followed by MDC staining, and observed using epifluorescence microscopy. Scale bar=50μm. Arrows indicate MDC-stained autophagosomes. (B) The number of MDC-stained autophagosomes per root section was counted in control conditions or after NaCl and mannitol treatment as above and the average number determined for 20 seedlings per treatment. (C) One week old GFP-ATG8e transgenic seedlings were transferred to control MS plates or to MS plates containing 0.16M NaCl or 0.35M mannitol for 6-8hs. GFP-ATG8e-labeled autophagosomes were visualized by fluorescence microscopy. Scale bar=50μm. Arrows indicate GFP-labeled autophagosomes.
Figure 2.3. RNAi-AtATG18a plants are hypersensitive to salt and osmotic stress treatment. (A) One-week-old WT and RNAi-AtATG18a seedlings were transferred to medium with 0.16M NaCl or 0.35M mannitol for 10 days. (B) One-week-old WT and RNAi-AtATG18a seedlings were transferred to MS+0.16M NaCl plates or MS +0.35M mannitol.
plates, followed by measurement of chlorophyll content (salt stress) or anthocyanin content (mannitol) per gram fresh weight at the indicated times, depicted relative to the zero day WT value. (C) WT and RNAi-AtATG18a seeds were germinated and grown in short-day conditions with regular watering every 2 days for 3 weeks, followed by salt and drought treatments for 5 weeks. For salt stress, the 3-week-old seedlings were watered with 0.16M NaCl solution every 2 days; for drought stress, water was withheld. In control conditions, watering was continued every 2 days throughout the experiment. (D) WT and RNAi-AtATG18a seeds were placed on control plates or plates containing 0.16M NaCl or 0.35M mannitol. The number of germinating seeds was counted every two days for eight days.
Figure 2.4. Autophagy is regulated by distinct pathways during different abiotic stresses. (A) One week old seedlings were transferred to medium lacking nitrogen for 4 days, and then transferred to liquid MS medium plus or minus 20μM DPI for 4h, or to liquid MS medium plus 0.16M NaCl or plus 0.35M mannitol plus or minus 20μM DPI for 4h. Autophagosomes were detected by MDC staining. (B) The number of MDC-stained autophagosomes per root section was counted in the presence or absence of imidazole for control, sucrose and nitrogen starvation, salt and osmotic stress conditions. The average number of autophagosomes was determined for 20 seedlings per treatment. (C) One week old AtATG18apro: GUS transgenic seedlings were transferred to medium lacking sucrose or nitrogen plus or minus 20mM imidazole for 4 days, or to liquid MS medium plus 0.16M NaCl or plus 0.35mM mannitol plus or minus 20mM imidazole for 6h. The seedlings were collected and submerged in GUS staining solution for 16h, destained in 70% ethanol for 16h and observed with a light microscope.
Table 2.1. Primers used for analysis of *AtATG18a*, *DREB2A*, *RD29A* and *SOS1* expression

<table>
<thead>
<tr>
<th></th>
<th>Forward primers</th>
<th>Reverse primers</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>AtATG18a</em></td>
<td>5'-TCGCGTCGACTCCTTCAAATCATTCTCCATG-3'</td>
<td>5'-TCGCTCTAGATTAGAAAACGTGAAGGCAGTTT-3'</td>
</tr>
<tr>
<td><em>DREB2A</em></td>
<td>5'-GGAGATGGGATTTATGATC-3'</td>
<td>5'-TTAGITCTCCAGATCCTATAGTA-3'</td>
</tr>
<tr>
<td><em>RD29A</em></td>
<td>5'-ATGGATCAAACAGAGAACCACCAC-3'</td>
<td>5'-CCATTTCTGTCCATCTTATC-3'</td>
</tr>
<tr>
<td><em>SOS1</em></td>
<td>5'-ATGACGACTG TAATCGACG GACGA-3'</td>
<td>5'-TGACAAACACCACCTGAGGAATAAT-3'</td>
</tr>
</tbody>
</table>

Supplemental Data

Figure S2.1. The effect of imidazole on autophagy. (A) For starvation conditions, 7-day-old WT seedlings were transferred to medium lacking nitrogen or sucrose for 4 days, and then transferred to liquid MS medium with or without 20mM imidazole for 4h. For salt and
osmotic stress treatment, one-week-old WT seedlings were transferred to liquid MS medium plus 0.16M NaCl or plus 0.35mM mannitol with or without 20mM imidazole for 4h. Autophagosomes were detected by MDC staining and fluorescence microscopy. (B) 7-day-old GFP-AtATG8e transgenic seedlings were treated as above and autophagosomes were detected using a fluorescence microscope.
Figure S2.2. The effect of ascorbic acid on autophagy. (A) For starvation conditions, 7-day-old WT seedlings were transferred to medium lacking nitrogen or sucrose for 4 days, and then transferred to liquid MS medium with or without 2 mM ascorbic acid for 4 h. For salt and osmotic stress treatment, one-week-old WT seedlings were transferred to liquid MS medium plus 0.16 M NaCl or plus 0.35 M mannitol with or without 2 mM ascorbic acid for 4 h. Autophagosomes were detected by MDC staining and fluorescence microscopy. (B) 7-day-old GFP-AtATG8e transgenic seedlings were treated as above and autophagosomes were detected using a fluorescence microscope.
**Figure S2.3. Protein oxidation analysis.** 7-day-old WT and RNAi-AtATG18a seedlings were transferred to solid MS medium containing 0.16M NaCl for the indicated days. Total proteins were isolated and derivatized by DNP followed by immunoblotting using DNP antibody. Molecular size markers are indicated at the left.
CHAPTER 3
TOR IS A NEGATIVE REGULATOR OF AUTOPHAGY IN
ARABIDOPSIS THALIANA

A paper published in *PLoS ONE*
Yimo Liu¹,³†, Diane C. Bassham¹,²,³‡

1. Interdepartmental Genetics Program
2. Plant Sciences Institute
3. Department of Genetics, Development and Cell Biology, Iowa State University, Ames, Iowa 50011, USA

3.1 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 \textit{AtATG} genes in the RNAi-\textit{AtTOR} plants. Unlike autophagy induced by starvation or salt stress, an NADPH oxidase inhibitor did not inhibit the constitutive autophagy in the RNAi-\textit{AtTOR} lines, indicating that \textit{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.

### 3.2 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 \textit{Arabidopsis thaliana}, many \textit{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 \textit{Arabidopsis}, an Atg18 homolog, AtATG18a, has been shown to be required for autophagosome formation during multiple environmental stress conditions and also leaf senescence [5]. \textit{AtATG18a} is expressed throughout the plant, with an increase in transcript level observed during conditions that upregulate the autophagy pathway [5,6]. In RNAi-\textit{AtATG18a} transgenic plants with reduced \textit{AtATG18a} transcript level, autophagosome formation was disrupted and the plants were more sensitive to autophagy-inducing stresses.
and displayed accelerated senescence [5,6,19]. 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 phosphatidylinositol 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 [28]. 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 dependent 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*.

### 3.3 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
transcript level. An RNAi-AtTOR construct was made by linking two inverted AtTOR gene specific regions with a 1kb 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 3.1A). 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.

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 3.1B), which is consistent with previous reports [40]. In yeast, TOR regulates growth and protein synthesis in response to nutrient availability [45,46]; therefore 10-day root length under nitrogen starvation conditions was also analyzed (Figure 3.1B). 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 3.1C). 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 3.1A) were germinated on MS plates and grown for one week, followed by MDC staining and fluorescence microscopy to visualize autophagy (Figure 3.2A). 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. Very 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-AtTOR 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 3.2B). 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-AtTOR plants.
We considered two possibilities to explain the constitutive presence of autophagosomes in RNAi-AtTOR plants: either the decreased expression of AtTOR leads to the constant formation of autophagosomes, or the RNAi-AtTOR 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 (conc A) was used to inhibit the degradation of autophagic bodies in the vacuole. Conc A increases the internal vacuolar pH and therefore inhibits vacuolar enzyme activities and prevents vacuolar protein degradation [48,49]. Autophagic bodies accumulate 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 (Figure 3.2C). 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 3.2B. 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), AtATG8h (At3g06420)] genes was also analyzed. WT and RNAi-2 seeds were germinated on control MS plates and grown for one week, RNA was extracted from 8 mm of the root tips where autophagy was seen and RT-PCR was performed. As shown in Figure 3.3A, in the RNAi-2 plants, where the AtTOR gene was partially silenced, the AtATG18a and AtATG9 transcript levels were increased compared with WT plants. However, the AtATG8b, AtATG8e, AtATG8f and AtATG8e genes didn’t show any differences. 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 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 AtATG8 genes tested compared with WT plants (Figure 3.3B). 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, AtATG18a: 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 3.3C). 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 *AtATG18a* gene is upregulated in the RNAi-2×*AtATG18apro: GUS* plants.

To further quantify the GUS staining results in figure 3.3C, 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-methyl umbelliferone (MU). *AtATG18apro: GUS* and RNAi-2×*AtATG18apro: 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 3.3D). 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. 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 3.4, 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 μM conc A or DMSO as a control for 12 h, and then observed by DIC microscopy (Figure 3.4B). 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 suggests 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 [5,6,19]. 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 iodinium) 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 3.5A). 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 3.5A), 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 3.5B). 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 inhibits 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 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 3.5C). 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.

3.4 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 3.1B), 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 3.2). The autophagy-related genes AtATG18a and AtATG9 were shown to have an increased transcript level in the RNAi-AtTOR lines (Figure 3.3A, 3.3B). AtTOR-regulated autophagy was dependent on AtATG18a, as RNAi-AtTOR×RNAi-AtATG18a plants do not show constitutive autophagy (Figure 3.4). Finally, an NADPH oxidase inhibitor did not inhibit autophagy in RNAi-AtTOR plants as it does under nutrient and salt stress conditions (Figure 3.5), 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 3.3C suggests an increased expression of *AtATG18a* 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 *AtATG18a* 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 EBP1 (ErbB-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 induction 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.
3.5 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 3.1). 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-AtTOR×RNAi-AtATG18a plants were generated by crossing RNAi-AtTOR plants with RNAi-AtATG18a plants [5]. RNAi-AtTOR×AtATG18apro: GUS plants were generated by crossing RNAi-AtTOR plants with AtATG18apro: GUS plants [19].

**RT-PCR analysis of AtTOR, AtATG18a 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 \textit{AtATG18a} and the \textit{AtATG8}s (\textit{b, e, f, h}), gene specific primers were used for \textit{AtTOR} and \textit{AtATG9} (Table 3.1). Gene specific primers used for PCR are also shown in Table 3.1. 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 \textit{AtTOR}, \textit{AtATG18a}, \textit{AtATG8}s and \textit{ATG9}, 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 four independent experiments.

\textbf{MDC staining and microscopy}

Wild-type and RNAi-\textit{AtTOR} seedlings were stained with MDC as previously described [47]. Seedlings were incubated with 0.05mM MDC for 10 mins, washed 3 times with phosphate buffered saline (PBS) and observed using a Zeiss Axioplan II compound microscope equipped with Axio Cam HRC digital imaging system (Carl Zeiss Inc., Göttingen, Germany). MDC fluorescence was visualized using a DAPI-specific filter and GFP fluorescence was visualized using a FITC-specific filter.

\textbf{Concanamycin A Treatment}

Seven-day-old seedlings grown on MS plates were transferred to MS liquid medium containing 1 \textmu M 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 differential interference contrast (DIC) microscopy.

\textbf{GUS staining}

Seedlings were collected and submerged for 16h in the following staining solution: Triton/ethanol stock (Triton X-100: ethanol: water; 1:4:5), 0.5 M KPO$_4$ 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-β-D-glucopyranoside in dimethyl sulfoxide (5: 470: 2: 2: 25). 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 5 min at 4°C at 14k 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 umbelliferone (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 4h.

### 3.6 Acknowledgements

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.

### 3.7 References


3.8 Figures and tables

Figure 3.1. 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 media and MS media lacking nitrogen (MS-N). C The fresh weight of WT, RNAi-2, RNAi-3 seedlings grown on MS media was measured at the stated times. *** indicates P<0.001; ** indicates P<0.01 and * indicates P<0.05. Error bars indicate standard error.
Figure 3.2. RNAi-AtTOR plants have constitutive autophagy. **A** Seven-day-old WT and RNAi-1 to RNAi-5 seedlings were stained with MDC and observed by fluorescence microscopy. Arrows indicate MDC-stained autophagosomes. **B** GFP-AtATG8e-labeled autophagosomes were visualized by fluorescence microscopy in 7-day-old GFP-AtATG8e and RNAi-2×GFP-AtATG8e seedlings. Arrows indicate GFP-labeled autophagosomes. Insets show an enlargement of the boxed areas. Scale bar=50 μm for main figures, 10 μm for insets. **C** Seven-day-old GFP-AtATG8e and RNAi-2×GFP-AtATG8e seedlings were transferred to liquid MS medium containing 1 μM concanamycin A (+concA) or DMSO (-concA) as a solvent control for 12 h, followed by both fluorescence and DIC microscopy. Scale bar=50 μm.
Figure 3.3. Some ATG genes are up-regulated in RNAi-AtTOR plants. A RNA was extracted from 8mm 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 AtATG18apro: GUS and RNAi-2×AtATG18apro: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 AtATG18apro: GUS and RNAi-2×AtATG18apro: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 replicates, with AtATG18apro: GUS 0 h value set as 1. ** indicates P<0.01 and * indicates P<0.05.
Figure 3.4. *AtTOR*-regulated autophagy is dependent on *AtATG18a*. A MDC staining was performed on seven-day-old RNAi-2 and RNAi-2×RNAi-*AtATG18a* seedlings and observed by fluorescence microscopy. The white arrow indicates MDC-stained autophagosomes. B Seven-day-old RNAi-2 and RNAi-2×RNAi-*AtATG18a* seedlings were transferred to liquid MS medium containing 1 µM concanamycin A (+concA) or DMSO (-concA) for 12 h, followed by DIC microscopy. Scale bar=50 µm.
Figure 3.5. NADPH oxidase inhibitor does not inhibit autophagy in RNAi-AtTOR plants. A 7-day-old WT and RNAi-2 seedlings were transferred to liquid MS media plus or minus 20 mM imidazole for 4 h, or WT seedlings were transferred to liquid MS media plus 0.16 M NaCl plus or minus 20 mM imidazole for 4 h. Autophagosomes were detected by MDC staining. Arrows indicated MDC-stained autophagosomes. B 7-day-old GFP-AtATG8e and RNAi-2× GFP-AtATG8e seedlings were transferred to liquid MS media plus or minus 20 mM imidazole for 4 h, or GFP-AtATG8e seedlings were transferred to liquid MS media plus 0.16 M NaCl plus or minus 20 mM imidazole for 4 h. Autophagosomes were detected by fluorescence microscopy via a FITC-specific filter. Arrows indicated GFP-labeled autophagosomes. Scale bar=50 µm. C Seven-day-old RNAi-2× GFP-AtATG8e seedlings were transferred to liquid MS medium containing 1 µM concanamycin A (+concA) or DMSO (-concA) as a solvent control for 12 h, or liquid MS medium plus 20 mM imidazole containing 1 µM conc A or DMSO for 12 h followed by both fluorescence microscopy and DIC microscopy. Scale bar=50 µm.

Table 3.1. Primers used for generating the RNAi-AtTOR construct and for RT-PCR analysis of AtTOR, AtATG18a, AtATG9 and AtATG8s.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward Sense</th>
<th>Reverse Sense</th>
<th>Forward Anti-sense</th>
<th>Reverse Anti-sense</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRV5'</td>
<td>AGCGGATATCATGTCCTACCTCGTCGCAATC-3'</td>
<td>XbaI5' -TCGCTCTAGACCAATCTCCGTCAACTCATC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EcoRI5'</td>
<td>AGCAGAATCATGTCCTACCTCGTCGCAATC-3'</td>
<td>BamHI5' -ACGCGAATCCCCAAATCTCCGTCAACTCATC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtTOR</td>
<td>5'-TCAGTCAGGCGAATATCATGTCCTACCTCGTCGCAATC-3'</td>
<td>5'-TATCCTAGCAAAATGATTTGAGGTAGC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtATG18a</td>
<td>5'-TCGCTCTAGATCATGTCCTACCTCGTCGCAATC-3'</td>
<td>5'-TCGCTCTAGATTAGAAAACTGAAGGCGGTTT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtATG9</td>
<td>5'-TCGCTCTAGATCATGTCCTACCTCGTCGCAATC-3'</td>
<td>5'-TCGCTCTAGATCATGTCCTACCTCGTCGCAATC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtATG8b</td>
<td>5'-TCGCTCTAGATCATGTCCTACCTCGTCGCAATC-3'</td>
<td>5'-TCGCTCTAGATCATGTCCTACCTCGTCGCAATC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtATG8e</td>
<td>5'-TCGCTCTAGATCATGTCCTACCTCGTCGCAATC-3'</td>
<td>5'-TCGCTCTAGATCATGTCCTACCTCGTCGCAATC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtATG8f</td>
<td>5'-TCGCTCTAGATCATGTCCTACCTCGTCGCAATC-3'</td>
<td>5'-TCGCTCTAGATCATGTCCTACCTCGTCGCAATC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtATG8h</td>
<td>5'-TCGCTCTAGATCATGTCCTACCTCGTCGCAATC-3'</td>
<td>5'-TCGCTCTAGATCATGTCCTACCTCGTCGCAATC-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 4
DEGRADATION OF THE ENDOPLASMIC RETICULUM BY AUTOPHAGY DURING ER STRESS IN ARABIDOPSIS THALIANA

A paper submitted to The Plant Cell
Authors: Yimo Liu1,2*, Junmarie Soto Burgos1, Yan Deng3, Renu Srivastava3, Stephen H. Howell1,3 and Diane C. Bassham1,2,3†

1. Department of Genetics, Development and Cell Biology, Iowa State University, Ames, Iowa, United States of America
2. Interdepartmental Genetics Program, Iowa State University, Ames, Iowa, United States of America
3. Plant Sciences Institute, Iowa State University, Ames, Iowa, United States of America

4.1 Abstract

In this study, we show a role for autophagy in the morphological changes in ER structure during ER stress in Arabidopsis thaliana. The ER stress agents tunicamycin and DTT trigger autophagy as demonstrated by increased production of autophagosomes and increased expression of the autophagy-related gene ATG18a. In response to ER stress, a soluble ER marker was shown to localize to autophagosomes and to accumulate in the vacuole upon inhibition of vacuolar proteases. Electron microscopy analysis showed that membrane lamellae decorated with ribosomes were observed inside the autophagosomes, demonstrating that portions of the ER are delivered to the vacuole by autophagy during ER stress. In

* Primary researcher and author
† Author for correspondence

Author contributions: Y.L., S.H.H. and D.C.B. designed research; J.S.B. conducted the initial experiments described in Figure 4.1 (B) (C); Y.D. generated the IRE1b-FLAG construct and complementation transgenic plants; R.S. provided the ire1a ire1b seeds and the CFP-HDEL construct; all the other experiments described in this chapter were conducted by Y. L.; Y.L., S.H.H. and D.C.B. analyzed data; Y.L. and D.C.B. wrote the paper.
addition, an ER stress sensor, \textit{IRE1b}, was found to be required for ER stress-induced autophagy. However, the IRE1b splicing target, bZIP60, does not seem to be involved, suggesting a novel signaling pathway to regulate ER stress-induced autophagy in plants. Together, our results suggest autophagy serves as a pathway for the turnover of ER membrane and its contents in response to ER stress in plants.

\section*{4.2 Introduction}

In eukaryotic cells, the endoplasmic reticulum (ER) is a central organelle for protein folding and maturation. To ensure correct protein function, polypeptide chains that enter the ER need to be successfully folded to their tertiary conformation and exported to their destination. However, in some cases the flux of unfolded proteins into the ER exceeds the capacity of the cellular folding machinery, resulting in the accumulation of unfolded or misfolded proteins, which is known as ER stress (Ron and Walter, 2007). To overcome this problem, the cell senses this imbalance and transmits a signal from ER to nucleus to activate the unfolded protein response (UPR) (Ron and Walter, 2007). The UPR usually involves the transcriptional up-regulation of some ER resident proteins, such as the BiP (Binding Protein) family of molecular chaperones to assist in proper folding of the misfolded proteins, and the attenuation of protein translation to reduce the load of nascent proteins entering the ER. The UPR-targeted genes can also activate the ER-associated degradation machinery (ERAD) to eliminate the misfolded proteins by delivery to the cytoplasm for proteasomal degradation (Mori, 2000; Ron and Walter, 2007). If ER homeostasis cannot be reestablished, cell death is triggered (Ron and Walter, 2007).

The first example of an ER to nucleus signaling pathway triggering the UPR was discovered in yeast, where the ER-resident transmembrane protein Inositol-requiring enzyme-1 (IRE1) binds to unfolded proteins (Gardner and Walter, 2011), thus sensing the ER stress, and splices the basic leucine zipper (bZIP) transcription factor HAC1 mRNA (Cox et al., 1993; Mori et al., 1993; Cox and Walter, 1996; Mori et al., 1996). The translation product from the spliced HAC1 mRNA regulates the transcription of targeted genes that possess UPR cis-
activating regulatory elements in their promoter regions (Mori et al., 1992; Kohno et al., 1993). Subsequently, three different classes of ER stress transducers were identified in animals, all of which are ER transmembrane proteins that sense ER stress through their luminal domain and transduce the signal to the nucleus (Ron and Walter, 2007). These include (1) the mammalian homolog of IRE1, which splices the mRNA of the bZIP-like transcription factor X-box binding protein 1 (XBP1) (Tirasophon et al., 1998; Yoshida et al., 2001; Calfon et al., 2002); (2) the activating transcription factor-6 (ATF6), which is transported to the Golgi to be processed by site 1 and site 2 proteases (S1P, S2P), followed by movement to the nucleus to activate targeted genes (Haze et al., 1999; Ye et al., 2000; Yoshida et al., 2000); and (3) the RNA-activated protein kinase (PKR)-like ER kinase (PERK), which regulates translation initiation factor-2a (eIF2a), thus attenuating translation (Harding et al., 2000). In plants, IRE1 homologs and two other ER stress sensors, bZIP28 and bZIP60, have been identified (Koizumi et al., 2001; Noh et al., 2002; Okushima et al., 2002; Liu et al., 2007; Iwata et al., 2008; Tajima et al., 2008; Iwata et al., 2009; Deng et al., 2011). In Arabidopsis, the IRE1 gene family has two members, IRE1a and IRE1b; both are located in the perinuclear ER (Koizumi et al., 2001). IRE1b is widely expressed throughout the plant whereas IRE1a is restricted to embryos and seeds (Koizumi et al., 2001; Noh et al., 2002). Like their yeast and animal counterparts, the plant IRE1s also possesses kinase and endoribonuclease domains (Koizumi et al., 2001). In response to ER stress, IRE1b splices the mRNA encoding bZIP60, which is a basic leucine-zipper domain containing transcription factor (Deng et al., 2011) in a manner similar to that of Hac1 in yeast or XBP1 in animals. The spliced bZIP60 mRNA is translated and activates the binding protein 3 (BIP3) gene (Deng et al., 2011). bZIP17 and -28 are additional transcription factors that localize to the ER membrane in unstressed conditions. In response to stress, they are cleaved and translocated to the nucleus, thus allowing activation of transcriptional responses (Liu et al., 2007).

While some aspects of the UPR signaling pathways have been elucidated in plants, morphological changes to the ER resulting from the UPR remain unclear (Urade, 2007, 2009; Moreno and Orellana, 2011). Previous research suggests that unfolded proteins can be degraded by both cytoplasmic ubiquitin-proteasome system (UPS)-dependent pathways and
UPS-independent pathways (Urade, 2007). However, the molecular mechanisms of UPS-independent ERAD-like pathways in plants are still unknown.

Autophagy functions as a degradation process in the recycling of cellular cytoplasmic contents and the removal of damaged proteins or organelles under unfavorable growth conditions. Upon induction of autophagy, a double membrane structure termed an autophagosome forms around cytoplasmic components. The autophagosome then delivers this cargo to the vacuole for degradation by hydrolases (Yang and Klionsky, 2009). The autophagy pathway is largely conserved from yeast to animals and plants. Studies in yeast cells demonstrated that autophagy is triggered by ER stress, and the IRE1-HAC1 signaling pathway is required for ER stress-induced autophagy (Yorimitsu et al., 2006). Electron microscopy analysis showed that autophagosomes contained ER lamellar membrane structures during ER stress (Bernales et al., 2006). Mammalian cells also showed the induction of autophagy under ER stress, and IRE1 is required for this process (Ogata et al., 2006). However, the XBP1 mRNA splicing by the IRE1 endoribonuclease activity is not involved in autophagy (Ogata et al., 2006). Instead, the IRE1 kinase activity-mediated c-Jun N-terminal kinase (JNK) pathway seems to be required for autophagy induction (Urano et al., 2000; Ogata et al., 2006). In addition, the ER was found to be one of the membrane sources for autophagosome formation (Hayashi-Nishino et al., 2009; Yla-Anttila et al., 2009). In plants, autophagy has been shown to be involved in responses to nutrient deprivation conditions, oxidative stress, salt and drought stresses, pathogen infection and senescence (Hanaoka et al., 2002; Liu et al., 2005; Xiong et al., 2005; Xiong et al., 2007b; Liu et al., 2009). Recent studies also suggest that autophagy can selectively degrade certain plant organelles, such as ribosomes (Hillwig et al., 2011) and chloroplasts (Ishida et al., 2008; Wada et al., 2009). However, whether autophagy contributes to ER membrane turnover and homeostasis in plants has not been addressed.

In this study, the potential involvement of autophagy and the morphological changes in ER structure during ER stress in plants were investigated. Tunicamycin and DTT were found to trigger autophagy in Arabidopsis, and an autophagy-related gene, ATG18a, is up-regulated during ER stress. Confocal and electron microscopy analysis showed that portions of ER are
engulfed by autophagosomes and delivered to the vacuole for degradation. Moreover, one of the ER stress sensors, IRE1b, but not the homolog IRE1a, was found to be required for autophagy induction by ER stress, although its bZIP60 mRNA splicing activity does not seem to be involved. Our results demonstrate that autophagy is involved in mitigating ER stress in plants and suggest that an alternative signaling pathway involving IRE1 activates autophagy in response to ER stress.

4.3 Results

ER stress induces autophagy in Arabidopsis

Tunicamycin (TM) and DTT are agents known to induce ER stress in Arabidopsis (Shorrosh and Dixon, 1991; D'Amico et al., 1992; Liu et al., 2007). To confirm that these two chemicals induce ER stress in our hands, Reverse Transcriptase-PCR (RT-PCR) was used to determine the expression level of BiP3 upon TM or DTT treatment. BiP3 is a molecular chaperone induced during the unfolded protein response (UPR), and thus can be used as an ER stress indicator (Koizumi, 1996; Noh et al., 2003; Tajima et al., 2008). 7-day-old Arabidopsis wild type (WT) seedlings grown on plates with MS (Murashige-Skoog) medium were transferred to MS liquid medium supplemented with 5µg/ml TM or 2mM DTT for 8 h, followed by RNA extraction and RT-PCR using BiP3 gene specific primers (Table 4.1, Figure 4.1A). The expression of BiP3 is barely detectable under control conditions, but increases significantly after TM or DTT treatment. This result confirms that 5µg/ml TM and 2mM DTT can be used to induce ER stress under our conditions.

Next, the effect of ER stress on autophagy in Arabidopsis was tested. 7-day-old WT seedlings, or transgenic seedlings expressing the autophagosome marker GFP (green fluorescence protein)-ATG8e, grown on MS plates, were transferred as before to MS liquid medium supplemented with TM or DTT for 8 h. ATG8e is an autophagosome membrane-associated protein that is conjugated to phosphatidylethanolamine (PE), and is used as an autophagosome marker (Yoshimoto et al., 2004; Contento et al., 2005; Thompson et al., 2005). Autophagosomes were detected in WT plants by staining with the autophagosome-
specific dye monodansylcadaverine (MDC) (Figure 4.1B) or in transgenic plants by visualization of the GFP-ATG8e marker (Figure 4.1C) (Conteto et al., 2005; Xiong et al., 2007b). In control conditions, autophagosomes were rarely seen, whereas numerous autophagosomes were visualized after TM or DTT treatment. These data suggest that autophagy is induced by both TM and DTT treatment, and therefore by ER stress.

Previously, *ATG18a* was identified as an essential gene for autophagy induction under several abiotic stresses. Thus, RNAi-*ATG18a* transgenic plants showed an autophagy-defective phenotype (Xiong et al., 2005; Xiong et al., 2007b; Liu et al., 2009). To investigate whether *ATG18a* is required for ER stress-induced autophagy, 7-day-old RNAi-*ATG18a* seedlings were subjected to ER stress as described above (Figure 4.1B). No autophagosomes were detected under control conditions, TM or DTT treatment; this indicates that ER stress-induced autophagy is dependent on the function of *ATG18a* and therefore most likely occurs via the classical autophagy pathway.

There are two possibilities to explain the increased number of autophagosomes seen under ER stress; either the ER stress leads to an increased rate of formation of autophagosomes, or the ER stress inhibits the degradation of autophagosomes in the vacuole. To distinguish between these two possibilities, the vacuolar H⁺-ATPase inhibitor concanamycin A (concA) was used to inhibit the degradation of autophagic bodies in the vacuole (Drose et al., 1993; Yoshimoto et al., 2004). ConcA inhibits trafficking of vacuolar proteins and prevents vacuolar protein degradation (Matsuoka et al., 1997; Yoshimoto et al., 2004). Autophagic bodies therefore accumulate in vacuoles after treatment with concA instead of being degraded (Yoshimoto et al., 2004). 7-day-old GFP-ATG8e seedlings grown on MS plates were transferred to liquid MS media containing 1µM concA or dimethyl sulfoxide (DMSO) as a solvent control, 5µg/ml TM or 5µg/ml TM+ 1µM concA for 12 h, then observed by both fluorescence microscopy using a FITC filter to visualize the GFP signal and differential interference contrast (DIC) microscopy to directly image autophagosomes (Figure 4.1D). In control conditions, the GFP signal was diffuse and the vacuole rarely contained any spherical structures in the DIC images. In the control+ concA condition, GFP-ATG8e-labeled puncta
and spherical structures were sometimes detected in the vacuole in certain cells since plants usually have a basal level of housekeeping autophagy (Inoue et al., 2006; Xiong et al., 2007a). After adding TM, several GFP-ATG8e-labeled puncta and spherical structures were observed, indicating the formation of autophagosomes. After adding both TM and concA, compared with the control+concA sample, the majority of cells showed a large number of GFP-ATG8e-labeled puncta and spherical structures in the vacuole which have been shown previously to be autophagic bodies (Yoshimoto et al., 2004). This indicates that after adding TM and concA, autophagic bodies accumulate inside the vacuole instead of being degraded and therefore autophagosomes were successfully formed and transferred to the vacuole. Together, these results show that the induction of autophagy by tunicamycin treatment is due to the increased formation of autophagosomes, rather than a deficiency in delivery to or fusion between autophagosomes and vacuoles.

Autophagy is regulated by either NADPH oxidase-dependent or -independent pathways, depending on the stress conditions (Liu et al., 2009). To determine whether ER stress-induced autophagy is dependent on NADPH oxidase, the NADPH oxidase inhibitor imidazole was used. 7-day-old WT plants grown on solid MS plates were transferred to liquid MS medium plus 2mM DTT or plus 2mM DTT with 20mM imidazole for 8h (Figure 4.1E). As demonstrated previously, autophagy could be induced by nitrogen starvation (–N treatment) and imidazole inhibited this induction (–N+20mM imidazole treatment) (Figure 4.1E) (Liu et al., 2009). Compared with control conditions, WT plants had substantially more autophagosomes when treated with DTT as shown above. The addition of imidazole together with DTT did not block autophagy induction, in contrast to the inhibition seen during nitrogen starvation; similar results were seen with the alternative NADPH oxidase inhibitor DPI (Figure 4.9). The effect of imidazole was also tested in RNAi-ATG18a plants (Figure 4.1F), and no autophagosomes were observed in control conditions, DTT treatment or DTT plus imidazole treatment. This result implies that ER stress-induced autophagy is regulated by an NADPH oxidase-independent pathway.
ATG18a gene is up-regulated under ER stress

Previous research has shown that several autophagy-essential genes increase in expression upon induction of autophagy (Rose et al., 2006). Under starvation, oxidative, salt and osmotic stresses, conditions that induce autophagy, the ATG18a gene has been shown to be up-regulated (Xiong et al., 2005; Xiong et al., 2007b; Liu et al., 2009). As we have determined that ER stress also induces autophagy, the expression level of ATG18a under ER stress was analyzed. In addition, ATG9 (At2g31260) and some members of the ATG8 gene family (ATG8b ATG8e, ATG8f, ATG8h) were tested since they are also essential for autophagy in Arabidopsis (Hanaoka et al., 2002; Yoshimoto et al., 2004). 7-day-old WT seedlings grown on MS plates were transferred to liquid MS medium or liquid medium supplemented with 2mM DTT for 8 h, followed by analysis by RT-PCR (Figure 4.2A). After DTT treatment, the transcript level of ATG18a increased compared to control conditions. However, no increases were observed for ATG9, ATG8b, ATG8e, ATG8f and ATG8e gene expression. 18S RNA was used as a control for equal RNA levels in each sample. To quantify the RT-PCR result, bands from three independent experiments were analyzed by densitometry, with the expression in the control set to 1 (Figure 4.2B). The mRNA level of ATG18a was significantly higher after DTT treatment than control conditions, but no significant difference was seen in expression of the ATG9 gene or any of the ATG8 genes tested.

As an alternative and more quantitative way to observe the expression of ATG18a, ATG18a::GUS transgenic plants were previously generated (Liu et al., 2009). In these plants, the GUS reporter gene was expressed under the control of the ATG18a promoter. We focused on the staining pattern in roots as a basal weak and variable staining pattern was observed in cotyledons and leaves (Liu et al., 2009), and as our assays for autophagy typically use root tissue. GUS activity is very low under control conditions in roots, but increases greatly upon induction of autophagy. Two independent transgenic lines were used in all experiments and behaved in the same way. To confirm the increase in ATG18a observed by RT-PCR, 7-day old ATG18a::GUS seedlings grown on MS plates were transferred to MS liquid medium or MS liquid medium supplemented with 2mM DTT for 8 h.
In control conditions, ATG18a pro: GUS roots have little visible GUS activity (Figure 4.2C). In contrast, GUS activity is evident throughout the roots after DTT treatment. This indicates that the ATG18a gene is up regulated under ER stress.

To further quantify the GUS staining results in Figure 4.2C, GUS activity in whole seedlings was measured using the fluorometric substrate 4-methylumbelliferyl-b-D-glucuronide (MUG) (Jefferson et al., 1987). MUG can be hydrolyzed by GUS to produce the fluorochrome 4-methylumbelliferone (MU). 7-day-old ATG18a pro: GUS seedlings grown on MS plates were transferred to MS liquid medium or MS liquid medium supplemented with TM or DTT for 8 h. In all conditions, GUS activity was evident, as indicated by an increase in fluorescence during the enzyme assay (Figure 4.2D). We interpret the GUS activity seen in control conditions as the basal weak GUS activity in aerial tissues, as these experiments were performed using whole seedlings. However, the GUS activity was greater upon TM or DTT treatment when compared with the control. At the end of the assay, the MU fluorescence in TM or DTT treatment was about 1.5 fold higher than that in control conditions; this difference is statistically significant (P<0.05). This again shows that the GUS activity was higher after TM or DTT treatment than the control conditions, which is consistent with the GUS staining results in roots. These results together indicate that the ATG18a gene is up-regulated during ER stress.

**ER is degraded via the autophagy pathway during ER stress**

One role of autophagy during ER stress might be to degrade regions of the ER, thereby turning over some of the ER membrane and contents of the ER. To investigate the physical relationship between the autophagy pathway and ER, the subcellular localization of both ER and autophagosome markers were analyzed in response to ER stress. GFP fused with the ER retention signal HDEL was used to label the ER (Batoko et al., 2000). To prevent vacuolar degradation, 1µM concA or DMSO as carrier control was added to the corresponding stress-inducing liquid MS media and incubated for 12 h in the dark. In the control, typical ER networks were observed, and there were few spherical structures in the vacuole in DIC images (Figure 4.3). The control+concA sample also showed typical ER patterns, although in
some cells small numbers of spherical structures were observed as mentioned above. To
induce ER stress, 7-day-old GFP-HDEL transgenic Arabidopsis seedlings were treated with
TM or DTT for 8h. With either treatment, the ER and the spherical structures were rarely
seen in the vacuole, similar to the control. However, upon TM+concA or DTT+concA
treatment, a fraction of the GFP-HDEL signal appeared as numerous dots, and the
accumulation of spherical structures greatly increased in the vacuole in the DIC images,
which morphologically resemble autophagosomes. For comparison, 7-day-old GFP-HDEL
plants grown on MS plates were transferred to MS plates lacking sucrose (-suc) or nitrogen (-N)
for 4 days to induce starvation stress, well-characterized autophagy activating conditions.
In the –suc and –N conditions, the GFP signal also appeared as an ER network and the
spherical structures were rarely seen in the vacuole. Interestingly, in –suc+concA and –
N+concA conditions, the increased accumulation of spherical structures was observed in the
DIC images, because autophagy is induced under starvation conditions (Contento et al.,
2005), whereas the GFP signal mainly showed an ER pattern instead of several dots. These
observations suggest that ER is transported to the vacuole via an autophagy-like pathway
during ER stress but not largely transported to the vacuole during nutrient starvation
conditions.

To test whether the spherical structures that deliver ER to the vacuole are autophagosomes,
leaf protoplasts obtained from 4-week-old GFP-HDEL plants were transformed with a
cerulean-ATG8e fusion construct, then incubated in the dark for 12h to allow gene
expression. Confocal microscopy was performed to visualize the subcellular localization of
both GFP-HDEL and cerulean-ATG8e (Figure 4.4A). (Note: the plant protoplasts expressing
only GFP-HDEL or cerulean-ATG8e displayed little fluorescence cross-talk between these
two signals.) In the controls, GFP-HDEL showed a typical cytoplasmic ER pattern, and the
cerulean-ATG8e gave a diffuse cytosolic signal. In the control+concA condition, most cells
also showed the GFP-labeled ER network and cytosolic cerulean signal, although a few cells
had GFP and cerulean puncta in the vacuole due to a basal level of housekeeping autophagy.
In the presence of TM, both the cerulean and the GFP labeled puncta appeared in most of the
cells, and some of the cerulean puncta and GFP puncta colocalized with each other. After
incubation with both TM and ConcA, in most of the cells numerous GFP puncta appeared as shown previously in planta, and the cerulean-ATG8e was also found in multiple puncta. Many of the structures labeled with these two markers colocalized and in the bright field, these puncta corresponded to small vesicles in the vacuole. Together, these data suggest that during ER stress, ER is delivered to the vacuole through an ATG8e-containing vesicle, presumably an autophagosome.

To further test the role of the autophagy pathway in delivering ER to the vacuole, similar experiments as described above were performed comparing WT and RNAi-ATG18a leaf protoplasts transiently expressing an CFP-HDEL fusion construct as an ER marker (Liu et al., 2007) (Figure 4.4B). Autophagosome formation is defective in RNAi-ATG18a plants, which thus can be used to test whether the loss of the autophagy pathway blocks ER transport to the vacuole during ER stress. In both WT and RNAi-ATG18a protoplasts, the CFP signal labeled an ER membrane network in control conditions. After addition of concA, most of the WT protoplasts showed an ER pattern and a few showed some CFP puncta, but almost all RNAi-ATG18a protoplasts observed displayed an ER pattern without CFP puncta. After adding TM, CFP puncta were observed in a majority of WT protoplasts, but not in RNAi-ATG18a protoplasts. After adding both TM and concA to the media, most of the WT protoplasts observed accumulated numerous CFP puncta inside the vacuole; however RNAi-ATG18a protoplasts still displayed the ER pattern, suggesting that delivery of ER to the vacuole is blocked when autophagy is defective. These results indicate that the delivery of ER to the vacuole is dependent on the autophagy-related gene ATG18a and this, when taken together with the co-localization between the ER marker and the autophagosome marker, suggests that the ER is delivered in autophagosomes.

Although it can be interpreted that the colocalization of ER puncta with an autophagosome marker indicates that ER is transported to the vacuole by autophagosomes, previous studies in animals showed that the autophagosome membrane can be derived from ER membrane (Hayashi-Nishino et al., 2009; Yla-Anttila et al., 2009) in which case one might observe the same pattern of colocalization. To clarify whether ER is delivered by the autophagosomes or
is a component of the autophagosome membrane during ER stress, electron microscopy was performed to examine the detailed structures of ER stress-induced autophagosomes (Figure 4.5). In the control condition, a few small vesicles were observed in both the vacuoles and the cytoplasm (Figure 4.5A). In the control+concA condition, there were a greater number of vesicles observed in the vacuole due to the inhibition of vacuolar degradation (Figure 4.5B). In response to TM treatment, numerous vesicles appeared in the cytoplasm, but the contents of the vesicles were hard to identify (Figure 4.5C). This might due to the fusion of autophagosomes with a smaller lysosome-like or endosome-like organelle, leading to the degradation of the contents in the autophagosomes before fusion with the vacuole (Rose et al., 2006; Toyooka et al., 2006; Bassham, 2007). Following treatment with TM+concA, a multitude of small vesicles were observed in both the cytoplasm and the vacuole (Figure 4.5D). Since the concA was used to inhibit vacuolar degradation, the contents inside the autophagosomes could be identified. These small vesicles contained a variety of cargos, some with unidentified cytoplasmic contents (Figure 4.5H), whereas many had membrane structures decorated with electron-dense ribosomes (Figure 4.5E, 4.5F, 4.5G), typical of ER. From this we conclude that ER membranes had been engulfed by autophagosomes. Together; these results imply that ER is transported to the vacuole for degradation via the autophagy pathway during ER stress.

**IRE1b is required for ER stress-induced autophagy**

In Arabidopsis, *IRE1* has been identified as an ER stress sensor. IRE1 senses ER stress and splices the mRNA encoding bZIP60, which is a basic leucine zipper domain-containing transcription factor implicated in the UPR in plants (Deng et al., 2011). There are two members of the IRE1 gene family in Arabidopsis, IRE1a and IRE1b (Koizumi et al., 2001), and IRE1b is primarily responsible for bZIP60 splicing in seedlings (Deng et al., 2011). To investigate whether ER stress-induced autophagy is activated via *IRE1* genes, the induction of autophagy was examined in *ire1a* or *ire1b* mutants compared with WT plants. 7-day-old *ire1a*, *ire1b* and WT plants grown on MS plates were transferred to MS liquid medium plus DMSO for 8h as a control, MS liquid media supplemented with 5µg/ml TM or 1mM DTT for 8h to induce ER stress, or MS plates deprived of sucrose (-suc) or nitrogen (-N) for 4 days to
induce starvation stresses, followed by MDC staining (Figure 4.6). In control conditions, all three genotypes showed very few autophagosomes. Both WT and ire1a plants showed elevated autophagy during TM or DTT treatment and starvation conditions (−suc, -N). Autophagy was also induced in ire1b during starvation conditions (−suc, -N), but autophagy induction was not observed in response to DTT or TM treatment. To further confirm the MDC staining result, concA was used to prevent vacuolar degradation of autophagosomes, which were visualized by DIC microscopy. 7-day-old ire1a, ire1b and WT plants were transferred to ER stress (+TM, +DTT) or starvation (−suc, -N) conditions as described above, plus 1µM concA or an equal amount of DMSO as a solvent control, followed by DIC image analysis (Figure S4.1). In the absence of concA, ire1a, ire1b and WT plants all displayed few spherical structures in the vacuole in almost all conditions tested. In the control+concA condition, all three types of plants showed a small number of spherical structures in the vacuole in some cells as described above. In the TM+concA or DTT+concA conditions, a substantial increase in spherical structures was observed in both WT and ire1a plants, but not in ire1b plants, which had a similar number of autophagosomes as in control+concA conditions. In −suc+concA or −N+concA conditions, all three types of plants showed an increased level of spherical structure accumulation compared with the control+concA condition. The DIC image analysis together with the MDC staining results indicate that IRE1b, and not IRE1a, is required for ER stress-induced autophagy but not for starvation-induced autophagy.

To further confirm the MDC and DIC results obtained in seedling roots, 4-week-old leaf protoplasts from WT, ire1a and ire1b plants were transformed with a GFP-ATG8e fusion construct to visualize the induction of autophagy under ER stress by confocal microscopy (Figure 4.7A). In the control, the fluorescence from GFP-ATG8e was diffuse in all three types of plant protoplasts. In the control+concA condition, most of the cells observed displayed a diffuse signal and occasionally GFP puncta were detected. In response to TM treatment, most of the WT and ire1a plants contained GFP-ATG8e labeled puncta, indicating the induction of autophagy, whereas the GFP signal was diffuse in ire1b, indicating no autophagosome formation. In the TM+concA condition, most of the WT and ire1a
protoplasts observed showed substantial accumulation of GFP puncta, but \textit{irel1b} plants behaved similarly as in the control+concA condition. These results again suggest that the ER stress-induced autophagy is dependent on \textit{IRE1b}.

Next, the role of \textit{IRE1b} in delivery of ER to the vacuole for degradation upon ER stress was analyzed using WT, \textit{ire1a} and \textit{irel1b} leaf protoplasts transiently expressing a CFP-HDEL fusion construct (Figure 4.7B). In control conditions, the ER pattern was typical in all three types of plant protoplasts. In the control+concA treatment, most of the cells displayed an ER pattern and occasionally CFP-labeled puncta were detected. In the presence of TM, most of the WT and \textit{irel1a} plants contained CFP labeled puncta, whereas \textit{irel1b} plants mainly showed a typical ER labeling pattern. In the TM+concA samples, most of the WT and \textit{irel1a} plants observed showed substantial accumulation of CFP-HDEL puncta, but \textit{irel1b} plants did not, behaving similarly as in control+concA conditions. These results imply that the delivery of ER to the vacuole is dependent on \textit{IRE1b} but not on \textit{IRE1a}. Together, the findings both in planta and in protoplasts suggest that \textit{IRE1b} is required for ER stress-induced autophagy.

To confirm that the loss of autophagy induction during ER stress in the \textit{irel1b} mutant is actually due to the lack of \textit{IRE1b} gene function, autophagy induction was tested in both \textit{irel1b} leaf protoplasts transiently expressing a FLAG-tagged \textit{IRE1b} construct (Figure 4.8A) and transgenic lines expressing the \textit{IRE1b} cDNA (\textit{IRE1b}-FLAG) in the \textit{irel1b} mutant background (Figure 4.8B). WT leaf protoplasts transiently expressing GFP-ATG8e displayed a diffuse GFP signal in control conditions, and GFP puncta were observed in the presence of TM as expected (Figure 4.8A). \textit{irel1b} leaf protoplasts transiently expressing GFP-ATG8e showed a diffuse GFP signal in both the control and after TM treatment. However, upon transformation of \textit{irel1b} leaf protoplasts with both \textit{IRE1b}-FLAG and GFP-ATG8e constructs, the GFP signal was diffuse in the control conditions but GFP puncta were seen in the presence of TM, similar to WT protoplasts. To confirm these results in planta, 7-day-old WT, \textit{irel1b} and \textit{irel1b/IRE1b-FLAG} complementation lines were transferred to liquid MS media supplemented with 5\textmu g/ml TM or DMSO, followed by MDC staining (Figure 4.8B). With TM treatment, both WT and \textit{IRE1b} complemented plants contained significantly more MDC-
labeled autophagosomes compared with control conditions, whereas in the *ire1b* mutant, few autophagosomes were detected in both control and in +TM conditions. Together, these results indicate that the defect for autophagy induction in *ire1b* in response to ER stress can be attributed to the loss of *IRE1b* gene function, rather than other defects in the autophagy pathway. This again suggests that ER stress-induced autophagy is dependent on the *IRE1b* gene.

**bZIP60 is not involved in regulating ER stress-induced autophagy**

As discussed above, IRE1b splices *bZIP60* mRNA to produce an active transcription factor, thus up-regulating the UPR genes in plants (Deng et al., 2011). To test whether regulation of ER stress-induced autophagy by IRE1b occurs via IRE1b splicing of *bZIP60*, the induction of autophagy was examined in an *bzip60* T-DNA insertion mutant (Deng et al., 2011). 7-day-old WT and *bzip60* plants grown on MS plates were transferred to MS liquid medium supplemented with 5µg/ml TM or DSMO as a solvent control, followed by MDC staining (Figure 4.9). Unexpectedly, the *bzip60* mutant showed constitutive autophagy even under control conditions. One explanation for the constitutive autophagy in the *bzip60* mutant is that the loss of *bZIP60* function causes constitutive ER stress, thus inducing autophagy. Alternatively, the loss in bZIP60 function may lead to general cellular stress, causing an increased level of basal autophagy. This complicated the testing of whether ER stress induces autophagy in *bzip60*, as autophagy seen upon TM treatment in *bzip60* could either be increased basal autophagy, or a mixture of the basal autophagy and TM induced autophagy. To distinguish between these two possibilities, the NADPH oxidase inhibitor DPI was used to inhibit the general starvation and salt stress-induced autophagy pathway (Liu et al., 2009). As shown in Figure 4.1, the addition of an NADPH oxidase inhibitor does not block ER stress-induced autophagy. In the presence of DPI, no autophagy was seen in *bzip60* (Figure 4.9), indicating that the constitutive autophagy observed in *bzip60* is inhibited by DPI and is therefore most likely a general stress response and unrelated to ER stress. After adding both DPI and TM to the media, WT plants still showed autophagy induction. Autophagosomes were also present in the *bzip60* mutant in the presence of DPI and TM, which suggests that after DPI inhibition of the enhanced basal autophagy, an alternative pathway for activation of
ER stress-induced autophagy was still active. These data indicate that autophagy can still be induced by ER stress in the bzip60 mutant and therefore that bZIP60 is not required for ER stress-induced autophagy. Thus, ER stress-induced autophagy is regulated by IRE1b but is not dependent on the downstream factor bZIP60.

Animal cells contain another two ER stress sensors, ATF6 and PERK, in addition to IRE1. Cells lacking ATF6 or PERK are capable of autophagy induction in response to ER stress (Ogata et al., 2006). In plants, bZIP28 may be functionally equivalent to ATF6, whereas PERK signaling has not been studied in detail (Liu et al., 2007; Tajima et al., 2008). To investigate whether bZIP28 is involved in ER stress-induced autophagy, an bzip28 KO mutant was tested for autophagy induction during ER stress. Similar experiments as described above for bzip60 were performed with 7-day-old bzip28 KO plants (Figure 4.9). The bzip28 KO plants displayed constitutive autophagy even in control conditions. This constitutive autophagy was inhibited by the addition of the NADPH oxidase inhibitor DPI, indicating that the constitutive autophagy seen in the bzip28 mutant was most likely a general stress response and unrelated to ER stress. After adding both DPI and TM to the media, autophagosomes were present in the bzip28 mutant, indicating that after the inhibition of general stress-induced autophagy, autophagy can still be induced by ER stress in the bzip28 mutant. These data imply that, like bZIP60, bZIP28 is not required for ER stress-induced autophagy.

4.4 Discussion

Although a number of studies have focused on UPR signaling pathways in plants, little is understood about ER morphology changes in response to ER stress, and the ubiquitin-proteasome system (UPS)-independent ERAD pathway is still unclear (Urade, 2007, 2009; Moreno and Orellana, 2011). Previously, plant autophagy had been shown to be involved in senescence, nutrient deprivation, oxidative stress, salt and drought stresses, and pathogen infection (Doelling et al., 2002; Hanaoka et al., 2002; Liu et al., 2005; Xiong et al., 2005; Xiong et al., 2007b; Liu et al., 2009). In this paper, we demonstrate that autophagy is
activated in the response to ER stress in plants. First, MDC staining and GFP-ATG8e transgenic plants showed autophagy induction after TM or DTT treatment. Second, the transcript level of a gene required for autophagy, ATG18a, increased during ER stress. Third, portions of ER are engulfed by autophagosomes and delivered to the vacuole for degradation. Together, this evidence implicates autophagy in ER turnover in response to ER stress. To investigate the upstream signaling pathway that activates ER stress-induced autophagy, a mutant lacking one of the ER stress sensors, IRE1b, was tested for autophagy induction upon ER stress. Leaf protoplasts transiently expressing CFP-HDEL or GFP-ATG8e indicated that IRE1b is required for ER stress-induced autophagy. To further characterize the IRE1b-dependent autophagy pathway, a mutant lacking the splicing target of IRE1b, bZIP60, was also analyzed. The bzip60 mutant was capable of inducing autophagy in response to ER stress, suggesting that ER stress-induced autophagy does not rely on the splicing activity of IRE1b.

Our data identified IRE1b as an upstream component of ER stress-induced autophagy in Arabidopsis. However, the detailed molecular mechanism of regulation of ER stress-induced autophagy is yet to be determined. In yeast, ER stress-induced autophagy is regulated through the IRE1 endoribonuclease activity towards HAC1 mRNA (Yorimitsu et al., 2006). In animals, IRE1 is also required for autophagy induction; however, the IRE1 kinase activity-mediated JNK pathway rather than the splicing activity towards XBP1 seems to control autophagy induction (Urano et al., 2000; Ogata et al., 2006). Our results showed that in plants, ER stress-induced autophagy is dependent on IRE1b, suggesting a conserved role for the IRE1 gene during autophagy induction from yeast to animals and plants. Similarly to in animals, autophagy does not depend on the IRE1b downstream splicing target bZIP60. As bZIP60 is the only known target of IRE1b ribonuclease activity, this raises the possibility that the kinase domain of IRE1b is responsible for the autophagy induction in response to ER stress, rather than its splicing activity. However, other functions of IRE1b in addition to the splicing of bZIP60 mRNA have not been discovered to date (Deng et al., 2011), and the JNK pathway found in animals has not been identified in plants. This suggests that a distinct, novel signaling pathway functions in activation of autophagy during ER stress in Arabidopsis.
To elucidate the potential role of IRE1 kinase activity in the autophagy pathway, further experiments are needed to identify its substrates and downstream signals. Intriguingly, the loss of XBPI in *Drosophila* causes constitutive autophagy (Arsham and Neufeld, 2009), similar to that of the *bzzip60* and *bzzip28* mutants observed here. The authors suggest that the absence of XBPI activity may lead to accumulation of unfolded proteins, triggering XBPI-independent UPR signaling. Whether this happens in plant cells still needs to be determined.

Target of rapamycin (TOR) has been shown to be a negative regulator of autophagy from yeast to animals and plants (Diaz-Troya et al., 2008; Liu and Bassham, 2010). Several studies have shown an interplay between ER stress and mTOR signaling in animals; for example, constitutive activation of mTOR leads to ER stress (Ozcan et al., 2008) and chaperone availability controls mTOR signaling (Qian et al., 2010). It was also suggested that ER stress induces autophagy through the inactivation of mTOR (Qin et al., 2010). However, whether TOR is associated with the control of autophagy during ER stress in plants is still unknown. A recent study in *Chlamydomonas* reported that the phosphorylation state of the BiP chaperone is regulated by TOR (Diaz-Troya et al., 2011). The authors showed that under ER stress when increased chaperone levels are needed, BiP protein is dephosphorylated, resulting in its activation. When protein synthesis was inhibited by down-regulating TOR activity, BiP was phosphorylated to its inactive form (Diaz-Troya et al., 2011). These results indicate a potential TOR function in interaction with the ER stress signal thereby regulating both protein synthesis and the autophagy degradation pathway. However, how exactly TOR senses ER stress, and whether IRE1 fits into this pathway, still needs to be determined.

Generally, autophagy is a non-selective process, however, organelle-specific autophagy has been identified in both yeast and animals (Reumann et al., 2010). For example, the selective degradation of peroxisomes (pexophagy) (Hutchins et al., 1999), mitochondria (mitophagy) (Kim et al., 2007), ribosomes (ribophagy) (Kraft et al., 2008), and ER (ER-phagy) (Bernales et al., 2006) have been reported. In plants, organelle-specific autophagy has not been studied extensively. Nevertheless, increasing evidence is emerging for organelle-specific autophagy in plants, such as the degradation of ribosomes (Hillwig et al., 2011) and RCBs (Ishida et al.,
However, whether the engulfment of ER by autophagosomes is a selective process is unknown. One possibility is that during ER stress, the ER begins to fragment, allowing it to be incorporated into autophagosomes non-selectively. Another possibility is that the autophagosome can recognize ER fragments containing misfolded proteins (Yorimitsu and Klionsky, 2007), therefore sequestering both the misfolded proteins and the ER membranes. Recent studies in animals suggest that p62/SQSTM1 (sequestosome 1) and NBR1 (neighbor of BRCA 1 gene) function as selective autophagy cargo receptors (Pankiv et al., 2007; Ichimura et al., 2008; Kirkin et al., 2009). Both p62 and NBR1 harbor an Atg8 interacting motif (AIM domain), which is a WXXL amino acid sequence that can be recognized by Atg8/LC3, and a UBA (Ub-associate) domain, which can interact with ubiquitinated proteins (Noda et al., 2010; Johansen and Lamark, 2011). It was proposed that upon ubiquitination of protein aggregates, the UBA domains of p62 and NBR1 recognize and bind to the ubiquitinated protein aggregates, then the AIM domains of p62 and NBR1 recruit Atg8/LC3, followed by the formation of autophagosomes (Johansen and Lamark, 2011).

Similar mechanisms have been reported in plants. NBR1 homologs in both Arabidopsis and tobacco have been identified, and they both interact with ATG8 (Svenning et al., 2011; Zientara-Rytter et al., 2011). Therefore it is possible that the autophagosome sequesters ER through identifying ER fragments containing protein aggregates; however, more evidence is required before this conclusion can be drawn.

In this study, we provide another link between organelle degradation and autophagy by showing that autophagy serves to turnover ER in response to ER stress in plants. Activities other than the splicing of bZIP60 by IRE1b may function as upstream events to regulate ER stress-induced autophagy. However, future experiments are needed to determine the downstream targets of IRE1b and the detailed regulation mechanisms in the ER stress-induced autophagy pathway.
4.5 Methods

Plant materials and growth conditions

*Arabidopsis thaliana* 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. Arabidopsis seedlings were grown under long-day conditions (16 h light) at 22°C on nutrient solid MS medium [Murashige-Skoog Vitamin and Salt mixture (Caisson, MSPA0910), 1% (w/v) sucrose, 2.4 mM MES (pH 5.7) and 0.8% (w/v) phytagar].

For tunicamycin and DTT treatment, 7-day-old seedlings grown on solid MS plates were transferred to liquid MS medium supplemented with 5µg/ml tunicamycin or 2mM DTT (Liu et al., 2007), or dimethyl sulfoxide (DMSO) as a solvent control for the indicated times in the dark.

For starvation treatment, 7-day-old seedlings grown on solid MS plates were transferred to MS plates lacking sucrose or nitrogen for an additional 4 days. Plants grown on sucrose starvation plates were incubated in the dark. If concA treatment (see below) was also required, the seedlings were then transferred to liquid MS medium lacking sucrose or nitrogen plus concA for 12 h in the dark.

For imidazole and DPI treatment, seedlings grown on solid MS plates were transferred to MS liquid medium plus or minus 20 mM imidazole or 20µM DPI for the indicated times. The solvent for DPI was DMSO; an equivalent volume of DMSO was added to controls.

For concA treatment, seedlings grown on MS plates were transferred to MS liquid medium containing 1 µM concA or DMSO as a solvent control for 12 h in the dark. The roots were mounted in water and then observed by fluorescence and differential interference contrast (DIC) microscopy.
**RT-PCR analysis of ATG8s, ATG9 and ATG18a**

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from each sample, followed by DNase I treatment (Invitrogen, Carlsbad, CA, USA). 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 BIP3, ATG18a and the ATG8 (b, e, f, h) genes; gene-specific primers were used for ATG9. Gene specific primers used for PCR are shown in Table 4.1. For BIP3 the PCR was run for 22 cycles with an annealing temperature of 55°C and extension time of 1 min. For the ATG genes, 28 cycles were used with annealing temperatures of 55, 55 and 60°C and extension times of 1, 1 and 4 min for ATG18a, ATG8s and ATG9, 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 3 independent experiments.

**MDC staining and microscopy**

*Arabidopsis* seedlings were stained with MDC as previously described (Contento et al., 2005). 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 compound microscope equipped with Axio Cam HRC digital imaging system (Carl Zeiss Inc., Göttingen Germany). MDC fluorescence was visualized using a DAPI-specific filter and GFP fluorescence was visualized using a FITC-specific filter.

**GUS staining**

Seeds 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-β-D-glucopyranoside in dimethyl sulphoxide (5:470:2:2:25). Plants were
washed once with 70% (v/v) ethanol, destained in 70% ethanol for 16 h and observed by light microscopy (Li et al., 2007).

**Fluorometric assay**

Fluorometric reactions for analysis of GUS activity were performed according to Jefferson et al. (Jefferson et al., 1987). 0.1 g of plant tissue was homogenized in 100 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 20,000×g. The supernatants were added to 1 mM MUG solution and at the indicated time points the reactions were terminated with 0.2 M Na₂CO₃ solution. Fluorescence was measured with a BIO-TEK Synergy HT multi-detection microplate reader, with excitation set at 360 nm and emission at 460 nm. The microplate reader was calibrated with freshly made 4-methyl umbelliferone (MU) standards of 0.5 nm, 5 nm, 25 nm and 50 nm.

**Generation of Cerulean-ATG8e construct**

The ATG8e cDNA was synthesized by RT-PCR from total RNA from 7-day-old seedlings grown on MS plates, using gene-specific primers (Table 4.1). The cDNA was sequenced for verification and ligated into the pAN578 vector (Rizzo et al., 2004). Protoplasts transformed with the Cerulean-ATG8e construct were observed with a CFP-optimized filter.

**Transient transformation of leaf protoplasts**

Arabidopsis leaf protoplasts were prepared and transformed according to Sheen (Sheen, 2002). 20 µg of plasmid DNA was used for each transformation. Protoplasts were incubated at room temperature in darkness for 12 h, with 40 rpm orbital shaking. Confocal microscopy was performed with a Leica confocal microscope using a 63×Leica oil immersion objective.

**Generation of IRE1b-FLAG construct and complementation transgenic plants**

IRE1b coding sequence was amplified from Col-0 cDNA using gene-specific primers. A 3X FLAG tag was added after the transmembrane (TM) domain of IRE1b by overlapping PCR. Primers used are listed in Table 4.1. IRE1b-N primers were used to amplify the first half of
the IRE1b gene up to and including the TM domain, IRE1b-C primers were used to amplify the second half of the IRE1b gene after the TM domain, and FLAG primers were used for the 3XFLAG tag. The IRE1b-3XFLAG DNA fragments were then ligated into the pSKM36 vector using Ascl/SpeI restriction sites. This construct was introduced into *Agrobacterium tumefaciens* by electroporation (Mersereau et al., 1990), and then into *ire1b* plants by Agrobacterium-mediated transformation using the floral dipping method (Clough and Bent, 1998). Transgenic plants were identified by kanamycin resistance. Individuals from the T2 generation were used for further studies.

**Electron microscopy analysis**

Electron microscopy was performed at the Iowa State University Microscopy and NanoImaging Facility. For transmission electron microscopy (TEM), samples were collected and fixed with 2% glutaraldehyde (w/v) and 2% paraformaldehyde (w/v) in 0.1M cacodylate buffer, pH 7.2 for 48 hours at 4°C. Samples were rinsed 3 times in 0.1M cacodylate buffer and then post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hour (room temp.) The samples were rinsed in deionized distilled water and enbloc stained with 2% aqueous uranyl acetate for 30 min, dehydrated in a graded ethanol series, cleared with ultrapure acetone, infiltrated and embedded using Spurr’s epoxy resin (Electron Microscopy Sciences, Ft. Washington, PA). Resin blocks were polymerized for 48 hours at 65°C. Ultrathin sections were made using a Reichert UC6 ultramicrotome (Leeds Precision Instruments, Minneapolis, MN), followed by collecting onto copper grids and counter-stained with 2% uranyl acetate in deionized distilled water for 30 min. Images were captured using a JEOL 2100 scanning and transmission electron microscope (Japan Electron Optic Laboratories, Peabody, MA).

**Accession Numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: At2g17520 (IRE1a), At5g24360 (IRE1b), At3g10800 (bZIP28), At1g42990 (bZIP60), At1g09080 (BiP3), At3g62770 (ATG18a),
At2g31260 (ATG9), At4g04620 (ATG8b), At2g45170 (ATG8e), At4g16520 (ATG8f) and At3g06420 (ATG8h).

Supplemental Data
Supplemental Figure 4.1. Autophagosomes do not accumulate in IRE1b roots in response to ER stress.

Author Contributions
Author contributions: Y.L., S.H.H. and D.C.B. designed research; Y.L., J.S.B., Y.D. and R.S. performed research; Y.L., S.H.H. and D.C.B. analyzed data; Y.L. and D.C.B. wrote the paper.

4.6 Acknowledgements
We thank Dr. Harry (Jack) T. Horner, Randall Den Adel and Tracey M. Pepper for assistance with the fluorescence, DIC and the electron microscopy and Margaret Carter for assistance with confocal microscopy. We also thank Dr. Ian Moore for the GFP-HDEL transgenic plant and Dr. Anthony L. Contento for generating the cerulean-ATG8e construct. This research was supported by grants IOB-0515998 and MBC-1051818 from the National Science Foundation to D.C.B.

4.7 References


Li, L., Ilarslan, H., James, M.G., Myers, A.M., and Wurtele, E.S. (2007). Genome wide co-expression among the starch debranching enzyme genes AtISA1, AtISA2, and AtISA3 in Arabidopsis thaliana. J Exp Bot 58, 3323-3342.


4.8 Figures and tables
Figure 4.1. Autophagy is activated in response to ER stress in Arabidopsis. (A) 7-day-old WT seedlings grown on MS plates were transferred to MS liquid medium supplemented with 5µg/ml TM, 2mM DTT, or DMSO as a solvent control for 8 h, followed by RNA extraction and RT-PCR. 18S RNA was a loading control. (B) 7-day-old WT or RNAi-ATG18a seedlings were transferred to MS liquid medium supplemented with 5µg/ml TM or 2mM DTT, or an equivalent amount of DMSO, for 8 h, followed by MDC staining and observed using epifluorescence microscopy. Arrows indicate MDC-stained autophagosomes. Scale bar = 50 µm. (C) 7-day-old GFP-ATG8e transgenic plants were transferred to MS liquid medium supplemented with 5µg/ml TM, 2mM DTT or DMSO for 8 h. GFP-ATG8e-labeled autophagosomes were visualized by fluorescence microscopy. Arrows indicate GFP-labeled autophagosomes. Scale bar = 50 µm. (D) 7-day-old GFP-ATG8e seedlings were transferred to liquid MS medium plus DMSO (cont), or plus 1 µM concanamycin A (+concA), or plus 5µg/ml TM (TM), or plus 5µg/ml TM with 1µM concanamycin A (TM+concA) for 12 h, followed by both fluorescence and DIC microscopy. Scale bar = 50 µm. (E) 7-day-old seedlings were transferred to liquid MS medium plus 2mM DTT plus or minus 20 mM imidazole for 8 h, or transferred to plates lacking nitrogen (-N) for 4 days and then to liquid MS medium plus or minus 20 mM imidazole for 8 h. (F) 7-day-old RNAi-ATG18a seedlings were transferred to liquid MS medium, or liquid medium plus 2mM DTT, or plus 2mM DTT with 20 mM imidazole for 8 h, followed by MDC staining. Arrows indicated MDC-stained autophagosomes. Scale bar = 50 µm.
Figure 4.2. *ATG18a* gene is up-regulated during ER stress. (A) 7-day-old WT seedlings grown on MS plates were transferred to MS liquid medium (cont) or MS liquid medium supplemented with 2mM DTT for 8 h, 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 three independent replicates, with the control condition value set as 1. * indicates P<0.05. Error bars indicate standard error. (C) 7-day-old *ATG18a*::GUS seedlings were transferred to MS liquid medium or MS liquid medium supplemented with 2mM DTT for 8 h. The seedlings were then 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) Extracts from 0.1g fresh weight of 7-day-old *ATG18a*::GUS seedlings treated with or without 2mM DTT, or with or without 5µM TM for 8 h 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 Na$_2$CO$_3$ followed by measurement of MU fluorescence. Data were collected from three independent replicates.
Figure 4.3. The localization of GFP-HDEL changes during ER stress in Arabidopsis roots. 7-day-old GFP-HDEL transgenic plants were transferred to liquid MS medium with or without 1μM concA for 12 h (control conditions). To induce ER stress, 7-day-old GFP-HDEL seedlings were transferred to liquid MS medium plus 2mM DTT or 5μg/ml TM with or without 1μM concA for 12 h. To induce starvation stress, seedlings were first transferred
to MS plates lacking sucrose or nitrogen for 4 days, then transferred to MS liquid medium lacking sucrose (-suc) or nitrogen (-N), with or without 1µM concA for 12 h. DMSO was used as a solvent control for all experiments. The plants were then observed with both fluorescence and DIC microscopy. Scale bar = 50 µm for main figures, 17 µm for insets.
Figure 4.4. Autophagy delivers ER to the vacuole during ER stress in Arabidopsis leaf protoplasts. (A) Leaf protoplasts obtained from 4-week-old GFP-HDEL plants were transformed with a cerulean-ATG8e fusion construct. For control conditions, the protoplasts were incubated in W5 solution with or without 1µM concA for 12 h. DMSO was used as a solvent control for all experiments. To induce ER stress, the protoplasts were incubated in
W5 solution plus 5µg/ml TM with or without 1µM concA for 12 h. Confocal microscopy was used to visualize the GFP and cerulean fluorescence. GFP-HDEL protoplasts lacking the cerulean-ATG8e construct were used as a GFP fluorescence control; WT protoplasts transformed with the cerulean-ATG8e construct were used as a cerulean fluorescence control. Scale bar=10µm. (B) Leaf protoplasts obtained from 4-week-old WT or RNAi-ATG18a plants were transformed with a CFP-HDEL fusion construct. For the control, the protoplasts were incubated in W5 solution with or without 1µM concA for 12 h. To induce ER stress, the protoplasts were incubated in W5 solution plus 5µg/ml TM with or without 1µM concA for 12 h. Confocal microscopy was used to visualize the CFP fluorescence. Scale bar=10µm.
Figure 4.5. ER membranes are engulfed by autophagosomes during ER stress. 7-day-old WT seedlings were transferred to MS liquid medium with (A) or without (B) 1µM concA, or MS liquid medium supplemented with 5µg/ml TM with (C) or without (D) 1µM concA for 12 h, followed by electron microscopy analysis. (E, F) Autophagic bodies with ribosome-decorated membranes inside. (G) Enlargement of a section indicated in (F). (H) Autophagic bodies with unidentified content inside. AB, autophagic bodies.
Figure 4.6. ER stress-induced autophagy is dependent on *IRE1b* function in *Arabidopsis* roots. 7-day-old WT, *ire1a*, and *ire1b* plants were transferred to MS liquid medium plus DMSO for 8h as a control, MS liquid medium supplemented with 5µg/ml TM or 2mM DTT for 8h to induce ER stress, or MS plates lacking sucrose (-suc) or nitrogen (-N) for 4 days to induce starvation stress, followed by MDC staining and fluorescence microscopy. Scale bar= 50µm.
Figure 4.7. ER stress-induced autophagy is dependent on IRE1b function in Arabidopsis leaf protoplasts. Leaf protoplasts obtained from 4-week-old WT, ire1a, and ire1b plants were transformed with GFP-ATG8e (A) or CFP-HDEL (B) fusion constructs. As a control, the protoplasts were incubated in W5 solution with or without 1 µM concA for 12 h. To induce ER stress, the protoplasts were incubated in W5 solution plus 5 µg/ml TM with or without 1µM concA for 12 h. DMSO was used as a solvent control. Confocal microscopy was used to visualize the GFP and CFP fluorescence. Scale bar= 10µm.
Figure 4.8. The defect in autophagy induction in ire1b during ER stress can be attributed to the loss of IRE1b gene function. (A) Leaf protoplasts obtained from 4-week-old WT or ire1b plants were transformed with the GFP-ATG8e fusion construct, or ire1b leaf protoplasts were transformed with both GFP-ATG8e and IRE1b-FLAG fusion constructs. For the control condition, the protoplasts were incubated in W5 solution. To induce ER stress, the protoplasts were incubated in W5 solution plus 5 µg/ml TM. (B) 7-day-old WT, ire1b, and ire1b/IRE1b-FLAG seedlings were transferred to MS liquid medium supplemented with 5µg/ml TM or DMSO as a solvent control for 8h, followed by MDC staining and fluorescence microscopy.
Figure 4.9. *bZIP60* and *bZIP28* are not involved in regulating ER stress-induced autophagy. 7-day-old WT, *bzip60* and *bzip28* plants grown on MS plates were transferred to MS liquid medium supplemented with 5µg/ml TM, or 20µM DPI, or 5µg/ml TM together with 20µM DPI, with DMSO used as a solvent control. MDC staining was performed to visualize autophagosomes. Scale bar= 50µm.
Supplemental figure

Figure S4.1. Autophagosomes do not accumulate in *IRE1b* roots in response to ER stress. 7-day-old WT, *ire1a*, and *ire1b* plants were transferred to conditions as described in Figure 6, plus 1µM concA or an equivalent amount of DMSO, followed by DIC microscopy. Scale bar= 50 µm for main figures, 17 µm for insets.
Table 4.1. Primers used for generating the cerulean-ATG8e construct, IRE1b-FLAG construct and for RT-PCR analysis of BiP3, ATG18a, ATG9 and ATG8s.

<table>
<thead>
<tr>
<th></th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerulean-ATG8e</td>
<td>5'-AGATCTATGAATAAGGAAGC-3'</td>
<td>5'-GCGGCCGCTTAGATTGAAGAAGCA-3'</td>
</tr>
<tr>
<td>BiP3 (36)</td>
<td>5'-GCTAAGAGTCACAATTGATTA-3'</td>
<td>5'-AACCGACTCAAAGACCTTC-3'</td>
</tr>
<tr>
<td>ATG18a</td>
<td>5'-TGGGCGAGACGTGAGCTG-3'</td>
<td>5'-TCGCTCATGAATTAGAATAACTGAAAGC-3'</td>
</tr>
<tr>
<td>ATG9</td>
<td>5'-TGCGACATGACGATGGGTCCTA-3'</td>
<td>5'-GGGCTCCTACTGAATCTGCGGTTGATG-3'</td>
</tr>
<tr>
<td>ATG8b</td>
<td>5'-AGATCTATGAGAAGAGCTC-3'</td>
<td>5'-TTAGATAGCTAGGAAAGATAATCC-3'</td>
</tr>
<tr>
<td>ATG8c</td>
<td>5'-AGATCTATGAAAAGACAT-3'</td>
<td>5'-TCTAGATAGATGAAGGAAGC-3'</td>
</tr>
<tr>
<td>ATG8f</td>
<td>5'-AGATCTATGAAAAGACAT-3'</td>
<td>5'-TCTAGATAGATGAAGGAAGC-3'</td>
</tr>
<tr>
<td>ATG8h</td>
<td>5'-AGATCTATGAAAAGACAT-3'</td>
<td>5'-TCTAGATAGATGAAGGAAGC-3'</td>
</tr>
<tr>
<td>IRE1b-N</td>
<td>5'-AAAAAGGCGCCGCCGAGATCTCCATATAACCTCCCCT-3'</td>
<td>5'-TTTGTCATCGTCGCTTGTAGTCTTTAATCGGTAAGAAAAGCGCA-3'</td>
</tr>
<tr>
<td>IRE1b-C</td>
<td>5'-GTGACTACAGACGATGACAACACAGACGCTTCCGATCAAGGAAC-3'</td>
<td>5'-AAAGAACTAGTGATACATACGATGTGGCTTATGAGTGGAAAG-3'</td>
</tr>
<tr>
<td>FLAG</td>
<td>5'-TGGGCGAGACGTGAGCTG-3'</td>
<td>5'-TTTAGTCTGTGAACCATTACGCTGAGATGAAAGAAG-3'</td>
</tr>
</tbody>
</table>
CHAPTER 5
CONCLUSIONS AND FUTURE WORK

5.1 General conclusions

This dissertation summarizes my work in investigating the autophagy-related pathways during stress conditions in Arabidopsis. These studies revealed that autophagy functions during a variety of environmental stresses, and is regulated by distinct signaling pathways in different conditions.

First, the studies in this dissertation have increased our understanding of autophagy function by demonstrating the involvement of autophagy during diverse abiotic stresses, such as ER stress, high salinity and osmotic stresses. Previously, plant autophagy has been suggested to recycle nutrients during senescence and starvation conditions, and degrade oxidized proteins during oxidative stress (5; 7; 17; 18). Here I show that autophagy-defective plants are more sensitive to high salinity and drought conditions, indicating a role for autophagy in removal of the damaged proteins or organelles produced in these environments. Heat stress is one of the most common environmental stresses encountered by plants, which is usually accompanied by the induction of ER stress (2; 6; 10). The unfolded protein response (UPR) is an ER stress response that mitigates the cellular damage (15). Although the molecular mechanism of the UPR has been studied extensively in plants, the morphological changes and the mechanism of degradation of ER is still unclear. I show that in Arabidopsis, ER can be engulfed by autophagosomes and delivered to vacuole for degradation via the autophagy pathway in response to ER stress. These results suggest autophagy is a general defense mechanism to protect plants from adverse environmental conditions.

Second, the studies in this dissertation have improved our understanding of the regulation of autophagy by identifying certain upstream components. The target of rapamycin kinase (TOR) has been suggested to negatively regulate autophagy in both yeast and animals (11; 12). However, due to the insensitivity of plants to rapamycin and the embryonic lethality
caused by disruption of the TOR gene in Arabidopsis, the study of TOR function in plant autophagy has been impeded. Here by generating RNA interference (RNAi)-AtTOR plants with reduced AtTOR transcript level, the role of AtTOR in plant autophagy was able to be investigated. Reduced AtTOR transcript level led to constitutive autophagy even under favorable growth conditions, implying TOR serves as a negative regulator of plant autophagy just as in yeast and animals. In addition, autophagy is regulated by distinct signaling pathways in different conditions. NADPH oxidase inhibitors block autophagy induction upon nutrient starvation and salt stress, but not during osmotic stress and ER stress, indicating that autophagy can be regulated by NADPH oxidase-dependent or -independent pathways (Figure 5.1). Moreover, AtIRE1b is identified as an upstream component of ER stress-induced autophagy, but not starvation-induced autophagy. We also suggested that the kinase activity rather than the endonuclease activity of AtIRE1b may be responsible for autophagy induction in response to ER stress, and this regulation is mediated by a novel pathway different from that of yeast and animals. These results provide useful information to further unveil the molecular mechanisms of autophagy induction in plants.

Finally, autophagy has been shown to play a vital role in health and diseases in animals (19). Understanding the autophagy mechanisms and pathways in plants can give us more information about its corresponding functions in mammals and yeast. It could also be helpful to agriculture since it shows how plants respond to stresses and senescence. Evidence already suggests that overexpression of ATG8 protein enhances plant growth and delays senescence under nutrient limitation conditions, possibly due to the elevation of the level of autophagy (14). Therefore crops can be engineered to have enhanced autophagy; plants that induce autophagy faster and more extensively could potentially tolerate the unfavorable environment better and senesce later.
5.2 Future directions

Identification of TOR substrates in the autophagy pathway
My studies suggested TOR functions as a negative regulator of plant autophagy. However, its downstream substrates that control the autophagy pathway still need to be identified. TOR works in complexes and some of its binding partners have been identified in Arabidopsis, including Raptor, which may recruit substrates and present them to TOR for phosphorylation (1; 3; 8). In Arabidopsis, two RAPTOR homologs have been identified, AtRAPTOR1A and AtRAPTOR1B (1; 3). Both Atraptor1A and Atraptor1B knockout mutants are available; therefore it is possible to study the AtTOR phosphorylation targets by comparing the phosphorylation profile of Atraptor mutant with WT plants. This can be achieved by staining protein gels with Pro-Q Diamond to detect phosphorylated amino acids (13; 16). Since TOR functions as a Ser/Thr protein kinase (9), TOR-regulated proteins can also be detected by anti-phospho-Ser (anti-P-Ser) and anti-phospho-Thr (anti-P-Thr) antibodies in both Atraptor mutant and WT plants (4). The proteins that show differences can be identified by mass spectrometry (MS) (13), followed by comparison with the Arabidopsis genome sequence. To determine whether TOR phosphorylation of the proteins identified is important in autophagy, plants harboring mutants that cannot be phosphorylated by TOR will be generated and tested for autophagy induction. If TOR phosphorylation of the identified protein is involved in autophagy induction, the corresponding mutant would not form autophagosomes after treatment with stress conditions (e.g. sucrose starvation).

Determination of the mechanism of AtIRE1b regulated autophagy in response to ER stress
Our studies suggest that AtIRE1b as an upstream component of ER stress-induced autophagy in Arabidopsis, and the kinase activity instead of the endonuclease activity of AtIRE1b may be responsible for the autophagy activation in response to ER stress. However, other functions of AtIRE1b in addition to the splicing of AtbZIP60 mRNA have not been discovered, and the detailed molecular mechanism of regulation of ER stress-induced autophagy remains to be determined. To investigate the regulatory mechanism of AtIRE1b in
the autophagy pathway, a mutant defective in AtIRE1b endonuclease activity yet retaining the kinase activity will be generated. MDC staining will be performed to test the autophagy induction in these plants upon ER stress. If autophagy activation is inhibited in these plants, the endonuclease activity is suggested to be required for the ER stress-induced autophagy. This implies that AtIRE1b splices a target RNA other than Atbzip60. To identify the new RNA targets, bioinformatic approaches can be performed to predict the potential RNA substrates through the RNA sequences and tertiary structures. The IRE1b splicing activity towards the RNA candidates can thus be further tested. If the autophagy activation is not affected in these plants, the kinase activity of AtIRE1b is suggested to participate in the ER stress-induced autophagy pathway. To further identify the downstream targets of IRE1b kinase domain, phosphorylation profiles of Atire1b mutant and WT plants can be compared as described above. In this way, the regulatory mechanism of ER stress-induced autophagy can be elucidated.

5.3 References

9. Hunter T. 1995. When is a lipid kinase not a lipid kinase? When it is a protein kinase. Cell 83:1-4
Figure 5.1 Autophagy is induced by two independent pathways. In nutrient starvation and salt stress, autophagy is most likely regulated by an NADPH oxidase-dependent pathway, involving ROS; whereas in osmotic stress, autophagy is regulated by an NADPH oxidase-independent pathway, which may not involve ROS as a signal.
ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. Diane C. Bassham. This dissertation could not have been written without her; she not only served as my advisor but also encouraged and financially supported me throughout my graduate studies. She provided me this valuable opportunity to work in her lab, taught me with her great patience, motivated me to research and most importantly opened the door of academic career to me, I truly respect her dedicated working attitude.

Of the many people who have been enormously helpful in the preparation of this dissertation, I am especially thankful to my POS committee members, Dr. Gustavo MacIntosh, Dr. David J. Oliver, Dr. Steven R. Rodermel, and Dr. Yanhai Yin, who supported me with generous advice and feedback.

I would also like to warmly acknowledge my former and current labmates, Dr. Yan Xiong, Dr. Anthony L. Contento, Dr. Ludmila Rizshsky, Dr. Sang-Jin Kim, Brice Floyd, Yunting Pu, and Rahul Roy, who are great people to work with and gave me lots of advice on both research and life. In addition, a special thanks to Xiayan Liu, Han Gao and all my other friends in ISU for their consideration and encouragement, their friendship made me feel like home in Ames.

I dedicate this dissertation to my parents and my husband Di Wu for their unconditional love and support in every way possible throughout the process of this course, this dissertation and beyond.