Interaction between regulation of autophagy, stress responses and growth in *Arabidopsis thaliana*

by

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The student author and the program of study committee are solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

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DEDICATION

This dissertation is dedicated to my dear grandma for raising me up and teaching me to be a strong and independent woman; to my parents for supporting me pursuing my academic career and teaching me to be a diligent and kind-hearted person; and to my husband, Xuan Lu, for completing my life, and me.

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ABSTRACT

Plants have evolved sophisticated mechanisms to balance between growth and stress tolerance upon changing environmental conditions. Autophagy is a critical process for recycling of cytoplasmic materials during nutrient remodeling and stress responses. Upon activation, the materials to be degraded are engulfed by a double-membrane vesicle called an autophagosome, which delivers the cargo to the vacuole for degradation and recycling. Studies in plants have revealed genes that are involved in the core machinery of autophagosome formation and delivery, and key regulators of autophagy. However, the upstream regulators of autophagy and the functions of autophagy in balancing growth and stress tolerance remain unclear. This dissertation summarizes my efforts in studying the regulation and functions of autophagy in *Arabidopsis thaliana*.

Previous studies identified a key regulator of autophagy, target of rapamycin (TOR), being a negative regulator of autophagy, and a positive regulator of plant growth. TOR has been suggested to be a nutrient sensor that activates autophagy during nutrient deficiency. Here we have assessed the extent to which TOR controls autophagy activation under abiotic stress. Through overexpression of TOR and activation of TOR activity by auxin, we have revealed that not only nutrient stress, but also salt and osmotic stresses regulate autophagy through a TOR-dependent pathway. In addition, oxidative stress and ER stress-induced autophagy are independent of TOR. Our results also have shown that auxin negatively regulates autophagy through the TOR-dependent pathway, providing a new mechanism of auxin-regulated stress tolerance in plants.

Another plant hormone that promotes plant growth is brassinosteroids (BRs). Our results identified a new link between the BR and TOR signaling through phosphorylation of

TOR by a BR regulated kinase Brassinazole-Insensitive 2 (BIN2). BRs were also characterized as negative regulators of autophagy, and BR-regulates autophagy and plant growth through the interaction with the TOR signaling pathway. This reveals a new mechanism of balancing plant growth and stress response through interaction between hormone signaling and regulation of autophagy.

We have also identified the TOR-independent pathway of autophagy regulation upon ER stress. ER stress is triggered when cells accumulate excessive unfolded and misfolded proteins, which then leads to the unfolded protein response (UPR). IRE1 is a dual-function protein kinase and ribonuclease, and one of the isoform, IRE1b, was shown to be dependent in the activation of autophagy upon ER stress. Here we have shown that the ribonuclease function of IRE1b is responsible for autophagy regulation, and we have identified three genes in the Regulated Ire1-Dependent Decay of Messenger RNA (RIDD) pathway that negatively regulate autophagy induction upon ER stress.

In summary, our results reveal that autophagy induced upon abiotic stresses is regulated through TOR-dependent and –independent pathways, and the TOR signaling pathway interacts with auxin and BR hormone signaling to regulate plant growth and stress responses. ER stress regulates autophagy is dependent of IRE1b ribonuclease function.

CHAPTER 1

GENERAL INTRODUCTION

The Functions and Regulation of Autophagy in Plant Cells

Modified from a combination of sections taken from:

A review published in *Plant Signaling & Behavior* [a]

Links between ER stress and autophagy in plants

Yunting Pu and Diane C. Bassham*

A book chapter published in *Plant Programmed Cell Death* [b]

To Live or Die: Autophagy in Plants. Autophagic Cell Death

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1.1 Abstract

Autophagy is a critical process for recycling of cellular materials during nutrient remodeling, senescence, and environmental stress response in eukaryotes. In plants, activated autophagy leads to formation of a double membrane vesicle named autophagosome, which delivers cargo to the vacuole for degradation and recycling. The mechanism of autophagosome formation has been well characterized, and studies on regulation of autophagy under different conditions have been emerging. Here, we summarize the current understanding of the mechanism, regulation and functions of autophagy in plants, and discuss future prospective of research on plant autophagy.

1.2 Introduction

Autophagy is a major pathway for delivery of proteins and organelles to lysosomes in mammalian cells or vacuoles in plant cells, where they are degraded and recycled. Three major types of autophagy have been described based on their mechanism; macroautophagy (Yang and Klionsky 2009), microautophagy (Mijaljica, Prescott et al. 2011) and chaperone-mediated autophagy (Orenstein and Cuervo 2010). Both macroautophagy and microautophagy can be either non-selective or selective, while chaperone-mediate autophagy is highly selective (Floyd, Morriss et al. 2012, Li, Li et al. 2012). To date, macroautophagy and microautophagy have been described in plants (Van der Wilden, Herman et al. 1980, Bassham 2007), and autophagy refers to macroautophagy hereafter. When autophagy is induced, the material that needs to be degraded begins to be surrounded by a double-membrane cup-shaped structure called a phagophore which is completed to form a double-membrane vesicle, the autophagosome. Upon delivery to the vacuole, the outer membrane of

the autophagosome fuses with the vacuole membrane, and the inner membrane with the cargo is degraded by vacuolar hydrolases (Liu and Bassham 2012) (Figure 1).

The molecular mechanism of autophagy was initially studied in yeast and animals, facilitating the discovery of components in the plant autophagy pathway. To date, more than 30 autophagy-related genes (ATG) that function in autophagy have been identified in yeast genetic screens, and many of them have also been identified in plants. Two types of markers have been used commonly to monitor autophagy flux in plants: monodansylcadaverine (MDC) staining, an acidotropic dye that stains acidic membrane compartments (Contento, Xiong et al. 2005), and green fluorescent protein (GFP)-ATG8 fusion proteins, since ATG8 participates in autophagosome formation via association with the autophagosome membrane (Yoshimoto, Hanaoka et al. 2004, Contento, Xiong et al. 2005, Li and Vierstra 2012). These markers have allowed the identification of conditions under which autophagy is activated in plants.

A key pathway for autophagy regulation is the target of rapamycin (TOR) signaling pathway. TOR is a PtdIns3K-related kinase that functions as a serine/threonine protein kinase, and it works as a component of larger complexes. In yeast and animals, there are two types of TOR complexes, mTORC1 and mTORC2, differentiated by distinct binding partners and functions (Loewith, Jacinto et al. 2002, Wedaman, Reinke et al. 2003). In plants, only homologs of mTORC1 components have been identified (Menand, Desnos et al. 2002, Díaz-Troya, Pérez-Pérez et al. 2008), including RAPTOR, which recruits substrates and presents them to TOR for phosphorylation (Hara, Maruki et al. 2002, Anderson, Veit et al. 2005, Deprost, Truong et al. 2005), and LST8, which stabilizes the complex (Díaz-Troya, Florencio et al. 2008, Moreau, Azzopardi et al. 2012). Previous studies indicated that TOR is both a

positive regulator of growth, and a negative regulator of autophagy in plants (Liu and Bassham 2010, Pérez-Pérez, Florencio et al. 2010), and it signals through phosphorylation of downstream substrates (Raught, Gingras et al. 2001, Ren, Qiu et al. 2011).

Autophagy has been extensively studied in humans and animals due to its important role in cancer, aging, immunity, and inflammatory responses (Yang and Klionsky 2010, Liu, Bi et al. 2012, Jones, Mills et al. 2013, Quy and Mizushima 2013, Levine and Klionsky 2017). In plants, autophagy is involved in the response to abiotic stresses and pathogen infection, in protein degradation during senescence, with a basal level of autophagy under normal conditions functioning as a housekeeping process (Hanaoka, Noda et al. 2002, Liu, Schiff et al. 2005, Bassham 2007, Bassham 2009, Hayward and Dinesh-Kumar 2011, Thomas 2013). Under stress conditions, autophagy is induced (Sláviková, Shy et al. 2005, Xiong, Contento et al. 2007), and these stresses include nutrient deprivation (Doelling, Walker et al. 2002, Thompson, Doelling et al. 2005, Xiong, Contento et al. 2005, Phillips, Suttangkakul et al. 2008), salt and drought stress (Liu, Xiong et al. 2009), oxidative stress (Xiong, Contento et al. 2007, Xiong, Contento et al. 2007), and ER stress (Liu, Burgos et al. 2012, Liu and Bassham 2013). Recently, autophagy has also been suggested interacts with plant hormone signaling and therefore is involved in regulation of plant growth and development (Zhang, Zhu et al. 2016, Nolan, Brennan et al. 2017). This dissertation presents the functions and regulation pathways for plant autophagy through revealing the regulation of autophagy upon different stress conditions, and interactions between autophagy and plant hormone signaling pathways.

1.3 Molecular mechanism of autophagy in plants

The core machinery of autophagy is mediated by ATG genes which were initially identified and studied in yeast and animals. Nearly 40 ATG genes have been identified in yeast, and many of which have homologs identified in plants (Yang and Bassham 2015). The identified genes can be divided into several functional groups (Yang and Klionsky 2010, Liu and Bassham 2012): the ATG9 cycling system, which is proposed to be responsible for the initiation of autophagosomes (Hanaoka, Noda et al. 2002, He and Klionsky 2007, Yoshimoto, Jikumaru et al. 2009, Yamamoto, Kakuta et al. 2012); an ATG1/ATG13 kinase group, which functions in ATG9 movement and autophagy induction (Kamada, Funakoshi et al. 2000, Xie and Klionsky 2007, Vigani 2011, Li and Vierstra 2012); two ubiquitin-like conjugation systems, that are responsible for the conjugation of ATG8 to the membrane lipid phosphatidylethanolamine (PE), with this ATG8-PE conjugation system being required for the complete formation of autophagosomes (Yoshimoto, Hanaoka et al. 2004, Yang and Klionsky 2009); and a phosphatidylinositol 3-kinase (PI3K) complex, which is required for the initiation of autophagosome formation in yeast and animals (Yang and Klionsky 2009, Liu and Bassham 2012), but is still poorly studied in terms of its function in plant autophagy.

One question remains for autophagy research is the source and initiation process of the phagophore membrane. Studies in animals have revealed several membrane sources of autophagosome membrane, including endoplasmic reticulum (ER), mitochondria and plasma membrane (Hayashi-Nishino, Fujita et al. 2009, Ylä-Anttila, Vihinen et al. 2009, Hailey, Rambold et al. 2010, Ravikumar, Moreau et al. 2010). ATG9 has been suggested involved in the initiation and elongation of autophagosome membrane in yeast and animals (Yamamoto, Kakuta et al. 2012). ATG9 cycles between different membrane sources and therefore it might

function to deliver lipids for phagophore expansion (He and Klionsky 2007, Yamamoto, Kakuta et al. 2012). In plants, ATG9 has also been suggested to be required for autophagy induction, since *atg9* knockout mutant has defects in autophagy induction, and has autophagy defective phenotypes such as early senescence and hypersensitive to stresses (Hanaoka, Noda et al. 2002). A very recent study suggested ATG9 is involved in autophagosome formation from ER membrane in Arabidopsis, suggesting ER is also a source of autophagosome membrane in plants (Zhuang, Chung et al. 2017). However, whether plant cells have other sources of autophagosome membrane remains unclear.

ATG1/ATG13 kinase group has also been suggested to function during the initiation of autophagosome formation through regulation of ATG9 movement between membrane sources (Kamada, Funakoshi et al. 2000, Mizushima 2010, Suttangkakul, Li et al. 2011). Study in yeast showed that ATG1 activates ATG8-PE conjugation for autophagosome formation through phosphorylation of ATG9 (Nair, Yen et al. 2012). Besides, ATG1/ATG13 kinase group also plays a role in regulation of autophagy (Suttangkakul, Li et al. 2011, Li, Chung et al. 2014). Defective ATG1/ATG13 kinase group through *atg13* knockout mutation leads to autophagy defective phenotypes in plants (Suttangkakul, Li et al. 2011). ATG1/ATG13 has also been shown to have a turnover mechanism through autophagy, and is mediated by ATG11 (Li, Chung et al. 2014). Studies in yeast and animals suggested ATG1/ATG13 complex is regulated through phosphorylation by TOR (Kamada, Funakoshi et al. 2000, Chang and Neufeld 2009). However, it is unclear whether in plants ATG1/ATG13 also acts downstream of TOR for autophagy regulation, or plants have developed a ATG1/ATG13 regulation pathway that is independent of TOR.

The most conserved machinery for autophagosome formation in yeast, animals and plants is the conjugation of ATG8-PE, which involves the function of two ubiquitin-like conjugation system (Liu and Bassham 2012) (Figure 2). The two ubiquitin-like conjugation system involves ATG8 and ATG12, and both proteins have ubiquitin-like features, and ATG8 conjugate to PE while ATG12 conjugate to ATG5 (Ohsumi 2001). The C-terminus of ATG8 protein is cleaved by a cysteine protease ATG4, and the exposed N-terminal glycine residue then covalently bound to an E1-like enzyme ATG7 (Yoshimoto, Hanaoka et al. 2004). ATG8 is then transferred to an E2-like enzyme ATG3, and eventually conjugate to PE. In the other conjugation system, ATG12 is also activated by the E1-like enzyme ATG7, and then transferred and covalently bound to ATG10, which mediates the conjugation of ATG12 to ATG5. The ATG12-ATG5 conjugates interacts with ATG16 covalently, and functions in the conjugation reaction to form ATG8-PE (Hanada, Noda et al. 2007, Chung, Phillips et al. 2010). The ATG8-PE conjugate then involves in the formation of autophagosome membranes, and ATG8 can also be cleaved from ATG8-PE by ATG4 for recycling. Knockout mutants of *atg5*, *atg7*, *atg10* or *atg12* all disrupt of autophagy induction or autophagosome formation, and hence lead to autophagy defective phenotypes like early senescence and stress hypersensitivity (Doelling, Walker et al. 2002, Thompson, Doelling et al. 2005, Phillips, Suttangkakul et al. 2008, Chung, Phillips et al. 2010). In animals, a non-canonical autophagy pathway that is independent of ATG5/ATG7 has been identified, however, whether plants also have a ATG5/ATG7 independent autophagy pathway is unknown (Nishida, Arakawa et al. 2009).

Another group that have been shown to function in the initiation of autophagosome formation if the PI3K complex, which is well characterized in yeast and animals (Yang and

Klionsky 2009). The PI3K complex functions on the phagophore membrane and recruits other PI3P binding proteins like ATG18 to the phagophore to facilitate autophagosome formation (Yang and Klionsky 2009). In yeast, the PI3K complex is composed of VPS34, a class III PI3K; VPS15, a serine/threonine kinase that is required for the membrane association of VPS34; ATG6, which co-localize with ATG8 but the function remains unclear; and ATG14, which links VPS34 and ATG6 (Yang and Klionsky 2009). In plants, homologs of VPS34, VPS15 and ATG6 have been identified, while ATG14 appears to be missing (Liu and Bassham 2012), suggesting plants might have developed their own mechanism of the PI3K system for autophagosome protein recruitment. ATG6 has been shown to regulate plant pollen germination, and disrupted ATG6 leads to autophagy defective phenotypes, reduced pathogen response and decreased fertility (Fujiki, Yoshimoto et al. 2007, Qin, Ma et al. 2007, Harrison-Lowe and Olsen 2008, Patel and Dinesh-Kumar 2008).

1.4. Regulation of autophagy in plants

Many regulators of autophagy have been identified in yeast and animals, however, many of them are not conserved or missing in plants (Michaeli, Galili et al. 2016). To date, several components have been identified in plants that regulate autophagy, including the TOR signaling complex, ATG1/ATG13 kinase complex, and IRE1 (Figure 3), although the extent to which these components function in distinct or overlapping pathways under different conditions remains not yet clear.

1.4.1 The TOR signaling pathway

The TOR signaling pathway is a key pathway for autophagy regulation and is probably the best-studied autophagy regulatory pathway identified so far in plants. In addition to autophagy, it is involved in the regulation of plant growth, stress resistance, mRNA translation, and cell wall formation (Deprost, Yao et al. 2007, Leiber, John et al. 2010). The major known component in the TOR signaling pathway in plants is the TOR complex itself, which is composed of TOR and its binding partners RAPTOR (regulatory associated protein of TOR) and LST8 (Lethal with Sec Thirteen 8). Genes encoding TOR complex homologs have been found in many plant species including *Arabidopsis*, rice, maize, soybean, and also in the model green alga *Chlamydomonas reinhardtii*. TOR is a highly conserved serine/threonine protein kinase that is inhibited by rapamycin via rapamycin binding to the FRB (FKBP12 and rapamycin binding) domain of both TOR and FKBP12 protein (Choi, Chen et al. 1996). Two distinct types of TOR complex exist in yeast and animals, TORC1 and TORC2, differentiated by distinct binding partners and functions (Loewith, Jacinto et al. 2002, Wedaman, Reinke et al. 2003). While TORC2 controls spatial cell growth, TORC1 modulates temporal cell growth by regulation of translation, and it negatively regulates autophagy (Loewith, Jacinto et al. 2002, Díaz-Troya, Pérez-Pérez et al. 2008). In plants, only TORC1 component homologs have been identified (Menand, Desnos et al. 2002), and this complex is therefore referred as the TOR complex here. The plant TOR complex contains TOR and two binding partners, RAPTOR, which recruits substrates and presents them to TOR for phosphorylation (Hara, Maruki et al. 2002, Anderson, Veit et al. 2005, Deprost, Truong et al. 2005), and LST8, which stabilizes the TOR complex (Díaz-Troya, Florencio et al. 2008, Moreau, Azzopardi et al. 2012).

TOR signaling functions via phosphorylation of substrates by the TOR complex (Raught, Gingras et al. 2001, Ren, Qiu et al. 2011). Ribosomal p70 S6 kinase (S6K) has been definitively identified as a substrate of TOR in plants (Mahfouz, Kim et al. 2006, Xiong and Sheen 2012). Several additional proteins have also been suggested to be substrates of the TOR complex, including: (a) Tap46, which is phosphorylated by TOR in plants and interacts with protein phosphatase type 2A (PP2A), a regulator of autophagy in yeast (Ahn, Han et al. 2011); (b) *Arabidopsis* Mei2-like1 (AML1), which interacts with RAPTOR *in vitro* (Anderson, Veit et al. 2005); (c) ErbB-3 epidermal growth factor receptor binding protein (EBP1), whose expression is correlated with that of TOR (Horváth, Magyar et al. 2006, Deprost, Yao et al. 2007); and (d) transcription factor E2Fa, which is phosphorylated by TOR *in vitro,* immunoprecipitates with TOR, and is involved in glucose sensitivity in root meristems (Xiong, McCormack et al. 2013).

TOR, the catalytic subunit within the TOR complex, is a PtdIns3-related kinase based on its sequence, but functions as a serine/threonine protein kinase. *TOR* is well conserved in yeast, animals and plants, and is widely expressed in embryos, endosperm, and primary meristems in *Arabidopsis* (Menand, Desnos et al. 2002). Disruption of *TOR* is embryo lethal (Deprost, Yao et al. 2007). Studies in yeast and animals show that TOR controls cell growth and protein synthesis in response to amino acids and growth factors during nutrient signaling, and it activates translation initiation by two regulatory pathways (Wedaman, Reinke et al. 2003). TOR phosphorylates S6K to activate translation of 5’ terminal oligopyrimidine tract mRNAs (Jefferies, Fumagalli et al. 1997). It also deactivates 4E-BP1 (eIF4E binding protein 1) through phosphorylation to in-turn activate the initiation factor eIF4E and the translation of mRNA containing a 5’ untranslated region (Sonenberg and Gingras 1998). In plants, TOR

is involved in the control of growth, development, and life span in response to nutrient and light energy status (Ren, Venglat et al. 2012, Caldana, Li et al. 2013, Xiong, McCormack et al. 2013).

RAPTOR is also highly conserved in eukaryotes. Two *RAPTOR* genes have been identified in *Arabidopsis*, *RAPTOR A* and *RAPTOR B*. *RAPTOR B* has a much higher level of expression than *RAPTOR A* (Anderson, Veit et al. 2005). In yeast and animals, RAPTOR recruits substrates such as S6K and 4E-BP1 and presents them to TOR to be fully phosphorylated (Hara, Maruki et al. 2002, Nojima, Tokunaga et al. 2003). In *Arabidopsis*, disruption of *RAPTOR* leads to severe defects in plant growth and development (Anderson, Veit et al. 2005, Deprost, Truong et al. 2005). RAPTOR has been shown to interact with S6K in response to osmotic stress signals, suggesting a role for RAPTOR in the TOR signaling pathway and plant stress responses (Mahfouz, Kim et al. 2006).

LST8 is suggested to be a binding partner of TOR and to stabilize the TOR complex (Díaz-Troya, Florencio et al. 2008, Moreau, Azzopardi et al. 2012). Two genes encoding LST8 have been identified in *Arabidopsis*, *LST8-1* and *LST8-2*, with *LST8-1* being much more highly expressed. Mutation of *LST8* results in defects in plant growth, flowering and metabolic adaptation to long days, similar to plants with reduced *TOR* expression, suggesting an important role for LST8 in plant growth regulation (Moreau, Azzopardi et al. 2012).

In addition to its role in growth regulation, the TOR signaling pathway negatively regulates autophagy in plants (Liu and Bassham 2010). When *TOR* transcript level was reduced by RNA interference in *Arabidopsis*, this led to constitutive autophagy (Liu and Bassham 2010). Some autophagy-related *ATG* genes were upregulated, including *ATG9* and *ATG18a* (Deprost, Yao et al. 2007, Liu and Bassham 2010). The regulation of autophagy by

the TOR signaling pathway depends on *ATG18a*, a gene required for autophagosome formation, indicating that the observed autophagy requires the classical autophagy components (Liu and Bassham 2010). TOR has also been shown to function as a negative regulator of autophagy in the model green alga *Chlamydomonas reinhardtii* (Pérez-Pérez, Florencio et al. 2010). In maize, the ortholog of *TOR* has been suggested to be involved in growth regulation, with S6K as substrate, although it is not clear whether TOR plays a role in the regulation of autophagy in maize (Reyes de la Cruz, Aguilar et al. 2004, Agredano-Moreno, Reyes de la Cruz et al. 2007).

In yeast, the TORC1 complex regulates the ATG1-ATG13-ATG17 complex in response to nutrient availability. Upon starvation, ATG13 is dephosphorylated, activating ATG1 and inducing autophagy (Kamada, Funakoshi et al. 2000, Yang and Klionsky 2009). In plants, the components downstream of the TOR complex remain poorly studied, but it is likely that they also include the ATG1 complex, which has been shown to be a regulator of autophagy in *Arabidopsis*.

1.4.2 ATG1/ATG13 kinase complex

ATG1 and ATG13 are components of the core autophagy machinery in yeast and function within a kinase complex, in which ATG1 is the catalytic subunit. This kinase positively regulates autophagy in response to nutritional status (Díaz-Troya, Pérez-Pérez et al. 2008, Mizushima 2010). In yeast, TOR hyper-phosphorylates ATG13 under nutrient-rich conditions, which decreases its affinity for ATG1, preventing their association and thus repressing the induction of autophagy. Under starvation conditions, inactivation of TOR leads to dephosphorylation of ATG13, enabling ATG1 to associate with ATG13 and

activating autophagy (Nakatogawa, Suzuki et al. 2009, Kamada, Yoshino et al. 2010). Interestingly, though these proteins are highly conserved, studies in animals reveal that ATG1 associates with ATG13 under all conditions, suggesting that the regulatory mechanism of ATG1/ATG13 is different in mammals compared with yeast (Lee, Kim et al. 2007). The ATG1/ATG13 complex activates autophagy via several subsequent steps, including the engagement of ATG9 in autophagosome formation and decoration of the phagophore with ATG8 and the VPS-34/ATG6/ATG14/VPS15 lipid kinase complex (Li and Vierstra 2012).

The ATG1/ATG13 kinase complex in yeast consists of ATG1, ATG13, ATG17, ATG29 and ATG31, but only ATG1 and ATG13 homologs have been identified in plants, including *Arabidopsis*, rice, maize and soybean, each with different numbers of homologs of each gene (Yoshimoto 2012). No ATG13 homolog has been found in *Chlamydomonas* (Avin-Wittenberg, Honig et al. 2012). Four *ATG1* homologs are present in *Arabidopsis*, including three full-length *ATG1* genes, *AtATG1a*, *AtATG1b* and *AtATG1c*, and a truncated version of *ATG1*. *AtATG1t*, which contains the kinase domain but not the regulatory domain and seems to be plant-specific (Suttangkakul, Li et al. 2011, Yoshimoto 2012). Two *ATG13* genes have also been identified in *Arabidopsis*, *AtATG13a* and *AtATG13b* (Yoshimoto 2012).

Previous studies on the ATG1/ATG13 kinase in *Arabidopsis* suggests that its function is conserved with that of other species (Suttangkakul, Li et al. 2011). ATG1a and ATG13a are reversibly-modified phosphoproteins, and their phosphorylation is associated with nutritional status. ATG1a is hyper-phosphorylated during starvation, while ATG13a is hypo-phosphorylated, suggesting that the ATG1/ATG13 kinase complex is a regulator of autophagy in response to nutrient conditions (Suttangkakul, Li et al. 2011).

A double mutant in *ATG13a* and *ATG13b* has the typical autophagy-related phenotypes of early senescence and hypersensitivity to nutrient deprivation. Biogenesis of autophagosomes or deposition of autophagic bodies is arrested in the *AtATG13* mutant upon nitrogen starvation, but ATG8 lipidation and ATG5-ATG12 conjugation are not, suggesting that the induction of the phagophore is independent of the ATG1/ATG13 kinase complex, but the complex may be essential for autophagosome closure or delivery to the vacuole (Suttangkakul, Li et al. 2011). The protein levels of ATG1a and ATG13a are strongly regulated by nutrient status, and their turnover is linked to autophagy. ATG1a associates with autophagic bodies and is delivered to vacuole for degradation, suggesting that the ATG1/ATG13 complex is a substrate of autophagy, and a negative feedback mechanism may exist to reduce ATG1/ATG13 complex levels after induction of autophagy (Suttangkakul, Li et al. 2011).

Taken together, the available information suggests that the ATG1/ATG13 kinase complex is a regulator of autophagy in plants, and meanwhile is also a target of autophagy in response to nutritional status. A feedback mechanism may therefore exist to regulate the amount of the complex and in turn to regulate autophagy in response to nutritional status.

1.4.3 IRE1

Autophagy is induced in plants under multiple stress conditions, including nutrient deprivation (Doelling, Walker et al. 2002, Thompson, Doelling et al. 2005, Xiong, Contento et al. 2005, Phillips, Suttangkakul et al. 2008), salt and drought stress (Liu, Xiong et al. 2009), heat stress (Zhou, Wang et al. 2013), oxidative stress (Xiong, Contento et al. 2007) and ER stress(Liu, Burgos et al. 2012).

ER stress is triggered when the amount of unfolded or misfolded proteins exceeds the capability of the degradation system in cells (Ron and Walter 2007, Vitale and Boston 2008), leading to a homeostatic response called the unfolded protein response (UPR) to assist proper folding or degradation of misfolded proteins. The ER folding machinery consists of a variety of molecular chaperones and other factors that assist in correctly folding polypeptides. One of the molecular chaperones in the ER is the binding protein BiP, a heat shock protein 70 that assists protein folding in the ER lumen by binding to nascent proteins when they enter the ER (Otero, Lizák et al. 2010). As addressed above, TOR has been shown to be a negative regulator of autophagy in *Chlamydomonas* (Pérez-Pérez and Crespo 2010). A recent study in *Chlamydomonas* revealed that BiP is a link between ER stress and the TOR signaling pathway (Díaz-Troya, Pérez-Pérez et al. 2011, Crespo 2012). However, whether the TOR signaling pathway is involved in autophagy regulation during ER stress is unknown.

The UPR is initiated by ER sensors located on the ER membrane. Inositol-requiring enzyme-1 (IRE1) is an ER sensor that activates a UPR signaling pathway in yeast (Cox and Walter 1996, Mori, Kawahara et al. 1996). Another two ER sensors are found in mammals, activating transcription factor 6 (ATF6) (Yoshida, Haze et al. 1998), and protein kinase RNA-like endoplasmic reticulum kinase (PERK) (Shi, Vattem et al. 1998). In plants, only IRE1 and ATF6 have been identified as ER sensors (Koizumi, Martinez et al. 2001, Liu, Srivastava et al. 2007), and only the IRE1 signaling pathway appears to be linked to regulation of autophagy (Ogata, Hino et al. 2006, Liu, Burgos et al. 2012).

IRE1 is highly conserved and functions as both a kinase and a ribonuclease (Cox and Walter 1996, Mori, Kawahara et al. 1996, Chen and Brandizzi 2013) (Cox and Walter 1996; Mori et al. 1996; Chen and Brandizzi 2013). Two *IRE1* homologs have been identified in

Arabidopsis, *IRE1a* and *IRE1b*, and they seem to have some distinct roles (Koizumi, Martinez et al. 2001, Deng, Humbert et al. 2011, Moreno, Mukhtar et al. 2012). When ER stress is triggered, IRE1 is activated by oligomerization and autophosphorylation (Korennykh, Egea et al. 2009). Activated IRE1 splices a mRNA encoding a membrane-associated basic leucine zipper transcription factor (bZIP60) in *Arabidopsis*, in an analogous manner to yeast and mammals (Nagashima, Mishiba et al. 2011). Spliced *bZIP60* mRNA is then translated, producing an active protein that is translocated into the nucleus and upregulates UPR genes such as *BIP* (Iwata and Koizumi 2005, Deng, Humbert et al. 2011). Defects in IRE1 cause enhanced cell death and inhibition of secretory pathway protein degradation upon ER stress, suggesting that IRE1 also plays a role in additional ER stress pathways (Mishiba, Nagashima et al. 2013). In addition to *Arabidopsis*, IRE1 and bZIP60 homologs have also been found in other plant species and show similar splicing mechanisms. In rice, one IRE1 homolog has been found, *OsIRE1* (Okushima, Koizumi et al. 2002), and two *bZIP60* homologs have been found, *OsbZIP74* and *OsbZIP50* (Hayashi, Wakasa et al. 2012, Lu, Yang et al. 2012). In maize, one *bZIP60* homolog has been found, *ZmbZIP60* (Li, Humbert et al. 2012, Wang, Zheng et al. 2012).

IRE1 is suggested to be a link between ER stress and autophagy in plants (Liu, Burgos et al. 2012, Liu and Bassham 2013, Pu and Bassham 2013). When ER stress is triggered by dithiothreitol (DTT) or tunicamycin, a mutant defective in *ire1b* is unable to form autophagosomes, suggesting *IRE1b* is required for the induction of autophagy under ER stress. Interestingly, mutations in either *IRE1a* or the only identified IRE1 target mRNA *bZIP60* have no effect on autophagy upon ER stress (Liu, Burgos et al. 2012). The ribonuclease function of IRE1 therefore may not be involved in autophagy regulation. As

mentioned above, IRE1 also functions as a protein kinase, suggesting that the kinase function of IRE1b may be the key for autophagy regulation. In animals, the c-Jun N-terminal kinase pathway acts downstream of IRE1 to activate autophagy, but there is no evidence for the existence of this pathway in plants (Howell 2013). Further research on IRE1b may reveal the mechanism of autophagy regulation by IRE1 under ER stress.

1.5 Functions of autophagy in plants

Studies in human have shown that many health and diseases are linked to autophagy, such as aging, neurodegenerative diseases, and cancer (Cai, Arikkath et al. 2016, Davidson and Heiden 2017). In plants, autophagy is induced during senescence, and plays an important role in nutrient recycling and remobilization and stress responses (Han, Yu et al. 2011, Liu and Bassham 2012). Autophagy has been shown to be involved in both abiotic stress and biotic stresses, and the regulation of autophagy is different between each condition.

1.5.1 Function of autophagy in abiotic and biotic stress tolerance

The best studied function of autophagy is the remobilization of nutrient, and thus autophagy plays an important role in response to nutrient deficiency. Autophagy is induced under sucrose or nitrogen starvation in plants with many ATG genes upregulated, and autophagy mutants are hypersensitive to nutrient deficiency (Doelling, Walker et al. 2002, Hanaoka, Noda et al. 2002, Thompson, Doelling et al. 2005, Xiong, Contento et al. 2005). Upon starvation stress, autophagy is activated and enhanced the recycling of organelles or proteins to assist survival of the cells. Autophagy is activated by nutrient deficiency through the TOR signaling pathway in yeast and animals, and the upstream stress sensor of TOR is

Snf1 in yeast and AMPK in animals. The homolog of AMPK and Snf1 in plants has been identified and is named SnRK1. However, whether SnRK1 sense nutrient signals to regulate TOR in plants remains unclear.

Another common stress conditions for plants are salt and drought stress. Both salt and drought stress leads to osmotic stress response, while salt stress also triggers ionic stress (Zhu 2016). Autophagy has been shown to be induced by salt and osmotic stress, and autophagy mutants are hypersensitive to both stress conditions (Liu, Xiong et al. 2009), suggesting autophagy is also important for salt and drought stress tolerance. In addition, salt and drought stress also increase the reactive oxygen species (ROS) in plant cells through NADPH oxidase. NADPH oxidase inhibitor blocks autophagy induced by salt stress but not osmotic stress, suggesting autophagy is regulated by salt and drought stress through both NADPH oxidase-dependent and –independent pathways.

Oxidative stress is triggered when cells accumulate excessive ROS and therefore cause damage to cellular components such as DNA, protein, and organelles. Oxidative stress triggered by H_2O_2 or Methyl Viologen leads to autophagy induction, and autophagy defective mutant showed accumulation of oxidized protein aggregates and was more susceptible to oxidative stress (Xiong, Contento et al. 2007). Oxidized protein has also been shown to be degraded through autophagy (Xiong, Contento et al. 2007), further suggesting that autophagy is critical for oxidative stress tolerance.

Recent studies have shown that ER is degraded by autophagy, and autophagy is also induced upon ER stress (Liu and Bassham 2013). ER stress is triggered when cells accumulate excessive unfolded or misfolded protein that is beyond the cells capacity (Howell 2013). ER stress signals through a series of transduction and eventually triggers genes to

enhance protein folding or degradation of misfolded protein, which is a process called unfolded protein response (UPR). Previous research have shown that autophagy induced by ER stress is dependent on a ER membrane chaperone Inositol-requiring enzyme-1b (IRE1b) (Liu and Bassham 2013), which has been discussed in the regulation of autophagy section.

Emerging evidences are showing autophagy is involved in immune response of pathogen infection. However, autophagy has been suggested both pro-survival and pro-death in response to different pathogen or different infection factors (Hofius, Schultz-Larsen et al. 2009, Yoshimoto, Takano et al. 2010, Hayward and Dinesh-Kumar 2011, Lenz, Haller et al. 2011). Pathogens are usually divided into two categories, biotrophs, which derive from host nutrient to survive and thus are less harmful to plants, and necrotrophs, which derive nutrient from damaged or dead host and thus have severe damage to plants. Both types of pathogens induce autophagy for immune responses. Recently, selective autophagy of degeradation of pathogens, which is termed xenophagy, has also been identified in plants (Hafrén, Macia et al. 2017), providing a new mechanism of defense for pathogen infection.

1.5.2 Function of autophagy in plant development and programmed cell death

Autophagy remains at a low basal level even under favorable conditions, which might due to its functions in degradation and recycling of protein aggregates or damaged organelles (Sláviková, Shy et al. 2005, Inoue, Suzuki et al. 2006, Yano, Suzuki et al. 2007). Autophagy mutants usually display growth and developmental defects such as reduced yield, reduced seed size or fertility, early flowering and senescence, suggesting autophagy is also important for regulation of plant growth and development, although many autophagy mutants are still able to have complete life cycles (Liu and Bassham 2012).

Autophagy plays an important role in nutrient remobilization, and therefore it is involved in regulation during senescence (Guiboileau, Sormani et al. 2010). Since most of the leaf nitrogen is contained in chloroplasts (Makino and Osmond 1991), autophagy has been shown to be involved in degradation of chloroplast stromal components through Rubisco-containing bodies (RCBs) (Ishida, Yoshimoto et al. 2008, Wada, Ishida et al. 2009, Izumi, Wada et al. 2010).

Nutrient remobilization is also involved in plants developing seeds during senescence (Guiboileau, Sormani et al. 2010), and therefore autophagy has also been shown to be involved in seeds development. In Arabidopsis, although autophagy mutants remains to have normal seeds development under normal growth conditions, ATG genes are differentially expressed during seeds development (Angelovici, Fait et al. 2009). Down regulation of ATG18a leads to delayed seeds development, suggesting autophagy might be critical for salt tolerance during seeds development (Liu, Xiong et al. 2009). Autophagy mutants showed defects in development of male gametophyte in rice, suggesting autophagy is required for reproductive organ development in plants (Kurusu, Koyano et al. 2014). A recent study also suggest autophagy is involved in nitrogen remobilization during seeds development in maize (Li, Chung et al. 2015).

Programmed cell death (PCD) is one mechanism of senescence. Autophagy has been suggested to be involved in programmed cell death through multiple mechanisms in plants (van Doorn and Woltering 2005). Severe stress conditions also triggers plants PCD, which might through the autophagic cell death process. PCD also happens in different cell developmental stages, including formation of xylem and phloem in plants (van Doorn and Woltering 2005). A previous study identified a small GTP-binding protein RabG3b is

responsive to salicylic acid (SA), and it has been shown to be a positive regulator of autophagy during xylem tracheary element (TE) differentiation in Arabidopsis (Kwon, Cho et al. 2010). This suggest that autophagy Is involved in PCD process of TE differentiation, and RabG3b might be a link between SA and autophagy.

SA has been a well-known plant hormone that is involved in multiple stress responses, especially immune response (Rivas-San Vicente and Plasencia 2011). Previous research showed that reducing SA level in autophagy mutants resulted in elimination of the early senescence, suggesting autophagy might regulates senescence through SA signaling (Yoshimoto, Jikumaru et al. 2009). Plants have developed a network with interactions between hormone signaling pathways to coordinate plant development and stress tolerance (Vanstraelen and Benkova 2012, Chaiwanon, Wang et al. 2016) A previous research also suggested the regulator of autophagy, TOR, is activated by auxin (Schepetilnikov, Dimitrova et al. 2013). Recent studies have identified a small GTPase ROP2 that is a downstream regulator of auxin, acts upstream of TOR to activate the TOR kinase activity (Deng, Yu et al. 2016, Li, Cai et al. 2017, Schepetilnikov, Makarian et al. 2017), suggesting an interaction between the TOR signaling pathway for autophagy regulation and the auxin signaling pathway for plant growth regulation. Besides, several recent studies showed that the brassinosteroids (BRs) transcriptional factor BES1/BZR1 are degraded through autophagy, and in particularly, BES1 is degraded through selective autophagy via interaction with ATG8, suggesting autophagy is involved in regulation of BR signaling pathway (Zhang, Zhu et al. 2016, Nolan, Brennan et al. 2017). In addition, brassinazole-insensitive 2 (BIN2) is a kinase that negatively regulates BR signaling in plants, and have been suggested to be a downstream effector of TOR, providing a new link between BR and TOR signaling for

balancing plant growth and stress tolerance. Furthermore, tryptophan-rich sensory protein/peripheral-type benzodiazepine receptor domain-anchored proteins (TSPOs) are membrane proteins that were identified as regulators of multiple stresses, and have been shown to be upregulated by ABA (Guillaumot, Guillon et al. 2009). Previous studies showed that TSPOs co-localized with ATG8, and were shown to be degraded through autophagy, suggesting TSPO might function as a link between ABA signaling and autophagy (Vanhee, Zapotoczny et al. 2011).

1.6 Future perspective

Autophagy has been first and better studied in yeast and animals. One key question in autophagy research is the membrane source of autophagosomes. In yeast and animals, sources of autophagosome membrane include ER, mitochondria, and plasma membrane. In plants, although ER has been identified as a membrane source (Zhuang, Chung et al. 2017), it is unknown whether plant also have other sources, and the mechanism of phagophore initiation and autophagosome expansion is also unclear. Future work is needed to solve these questions.

Although most of the genes in autophagy machinery and regulation are conserved, many genes and regulators are missing in plants. For example, AMPK is the nutrient and energy sensor in animals that regulates TOR activity through TSC complex (Inoki, Zhu et al. 2003), which is missing in plants. Plants might have developed their own mechanism of nutrient and other stress sensing for regulation of autophagy, but further investigation is needed to identify other regulators.

Emerging evidences are showing plants have selective autophagy that degrades protein aggregates and specific organelles including chloroplast, mitochondria, ER, peroxisome, and other organelles (Floyd, Morriss et al. 2012). Selective autophagy usually involves specific adaptors that targets components that needs to be degraded, and bound to ATG8 though the ATG8 interaction motif (AIM) for autophagosome formation. Some adaptors have been identified in plants, including NBR1 and RPN10 (Svenning, Lamark et al. 2011, Zhou, Wang et al. 2013, Marshall, Li et al. 2015). However, further investigation is necessary to find more adaptors for selective autophagy in plants, and to study the functions of each types of selective autophagy.

Although autophagy mainly functions as degradation of cellular materials under stress conditions, autophagy might be also involved in regulation of other biological processes. Increasing evidences are suggesting interactions between autophagy or the regulation pathways of autophagy and plant hormone signaling pathways. Studies of the function of autophagy in regulation of other signaling transduction pathways may help us better understand how plants balance stress tolerance and growth under changing environmental conditions. This may also provide new approaches to increase crop yield and stress tolerance through modulation of autophagy.

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1.8 Figures and tables

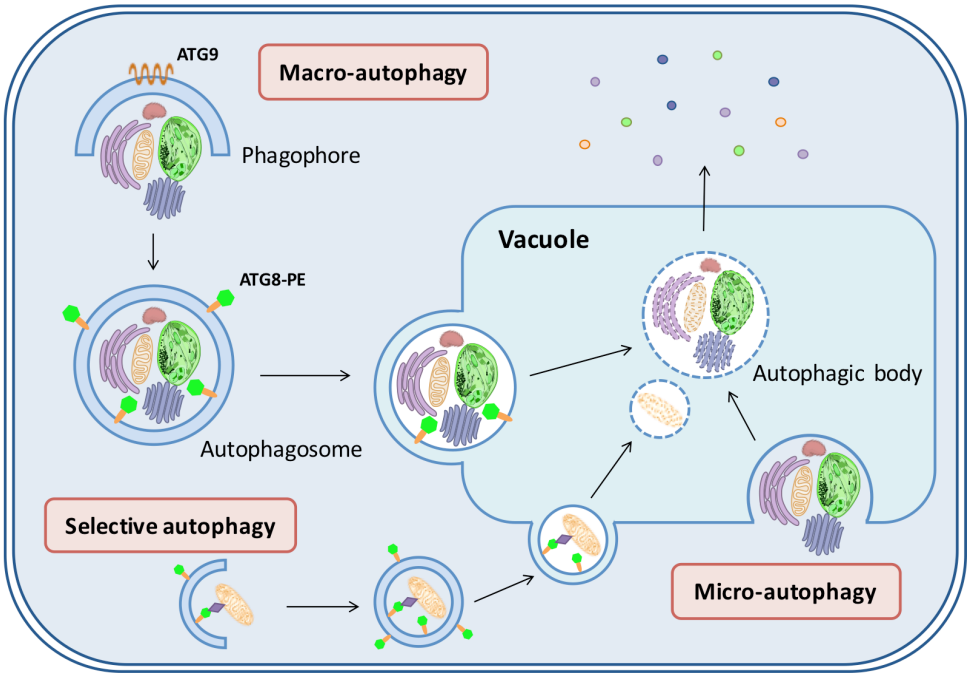

Figure 1. Schematic of autophagy morphology in plants

Major autophagy pathways in plants. When macroautophagy is triggered, the formation of a double-membrane cup-shaped structure called the phagophore is initiated with the aid of ATG9 and begins to surround organelles or protein aggregates to be degraded. Closure of the phagophore forms a double-membrane vesicle called an autophagosome. ATG8 is required for autophagosome formation and expansion. ATG8 is anchored to the autophagosome membrane by conjugation to phosphatidylethanolamine (PE) and thus is used as a marker to visualize autophagosomes. Autophagosomes carry the cargo to the vacuole, where the outer membrane of the autophagosome fuses with the vacuolar membrane, releasing the inner membrane and the cargo into the vacuole as an autophagic body. Autophagic bodies are degraded in the vacuole to small molecules and exported back to the cytosol for reuse. Selective macroautophagy is a specialized form of macroautophagy in which a receptor (purple diamond) recognizes both a specific organelle or protein and also ATG8. The specific cargo is thus recruited to the autophagosome and delivered to the vacuole for degradation. In microautophagy, the tonoplast directly engulfs the cargo, forming an autophagic body that is degraded inside the vacuole.

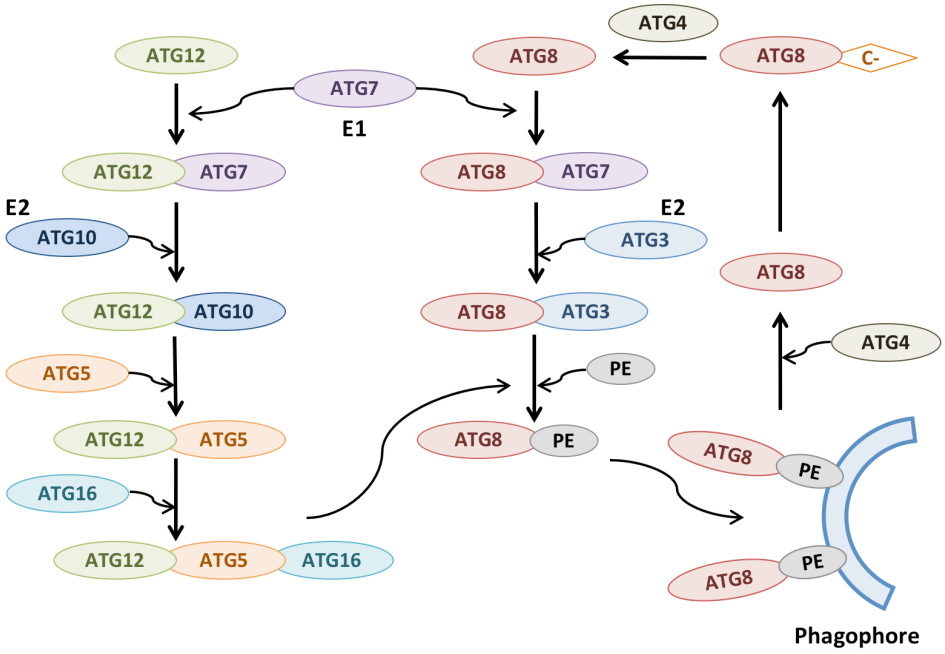

Figure 2. Molecular mechanism of autophagy in plants

Two ubiquitin-like conjugation system for ATG8 lipidation in Arabidopsis. The C-terminus (Orange diamond C-) of ATG8 is cleaved by ATG4 and associate with ATG7, an E1-like enzyme. Associated ATG8 is transferred to ATG3, an E2-like enzyme, and eventually conjugated to phosphatidylethanolamine (PE). ATG12 also associate with ATG7, followed by transferring to the E2-like enzyme ATG10, and eventually conjugated to ATG5. The ATG5/12 conjugate then interact with ATG16, and together function as an E3-like complex that is involved in ATG8 lipidation and phagophore expansion. ATG8-PE conjugate is recycled by ATG4 cleavage.

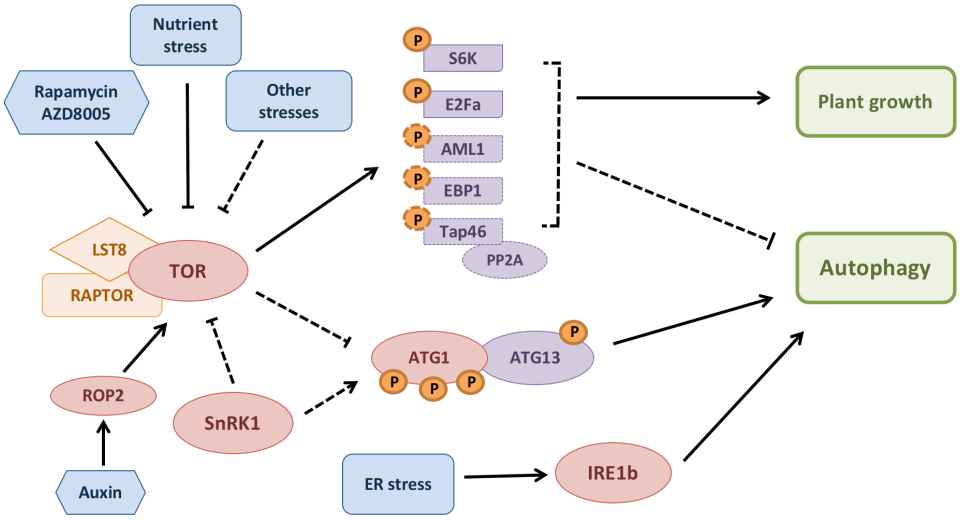

Figure 3. Regulation pathways of autophagy in plants

Three major autophagy regulation pathways in plants, the TOR signaling pathway, ATG1/ATG13 kinase complex, and IRE1b. TOR function as a complex with binding partners RAPTOR and LST8. TOR activity is downregulated upon stress conditions and drug treatment such as rapamycin and AZD8055. Identified (solid outline) and predicted (dashed outline) substrates that are phosphorylated by TOR kinase (Orange circle P) include Ribosomal p70 S6 kinase (S6K), transcription factor E2Fa, Mei2-like1 (AML1), ErbB-3 epidermal growth factor receptor binding protein (EBP1), and Tap46, which interacts with protein phosphatase type 2A (PP2A). S6K and E2Fa are involved in regulation of plant growth and development. Tap46 and PP2A have been shown to regulate autophagy in plants, however, whether other substrates are involved in autophagy regulation remains unclear (dashed lines). SnRK1 is the homolog of mammalian AMPK, and is suggested to be upstream regulator of TOR in plants. TOR is also activated through phosphorylation by a small GTPase Rho-related protein 2 (ROP2) upon auxin signaling. ATG1/ATG13 complex is suggested to be a positive regulator of Autophagy. Studies in yeast and animals showed that ATG1/ATG13 is regulated by TOR or directly by AMPK, but it remains unclear whether the mechanism remains the same in plants. Autophagy is also induced by Endoplasmic Reticulum (ER) stress through an inositol-requiring enzyme-1b (IRE1b) dependent pathway.

1.9 Dissertation organization

This dissertation summarizes my research on function and regulation of autophagy in plant growth and stress responses.

Chapter 1 is a general introduction and review on current understanding of autophagy in plants. I wrote this section with combination of my previous published reviews. My contribution to this chapter is 100%.

Chapter 2 describes the activation of autophagy in response to stresses is regulated through TOR-dependent and –independent pathways. The TOR signaling pathway is involved in autophagy induction upon nutrient deficiency, salt and osmotic stress, while oxidative and ER stress induced autophagy is independent of TOR. We also showed that auxin can be used as a TOR activator, and auxin negatively regulates autophagy under nutrient deficiency, salt and osmotic stress conditions. Xinjuan Luo performed the experiments on auxin and analyzed data shown in figure 4, and I took the confocal images. I performed the remaining experiments and wrote the manuscript, and my total contribution to this chapter is about 80%.

Chapter 3 reports a new interaction between Brassinosteroids (BRs) and the TOR signaling pathway in regulation of plant growth and autophagy. We found BRs negatively regulates autophagy through phosphorylation of TOR by Brassinazole-insensitive 2 (BIN2) kinase, suggesting a new mechanism of plants balancing growth and stress response through hormone signaling and autophagy. Trevor Nolan performed the gene expression analysis shown in Figure 1, helped cloning and performed *in vitro* protein assays shown in figure 4, prepared protein sample for mass-spectrometry in table 1, and helped revision of the manuscript. Gaoyuan Song performed the mass-spectrometry experiment and analyzed data

shown in table 1. Dr. Justin Walley wrote the methods for mass-spectrometry experiment. I performed the remaining experiments and wrote the manuscript, and my total contribution to this chapter is about 60%.

Chapter 4 reports a new mechanism for IRE1b-dependent pathway of autophagy activation upon ER stress. We characterized that IRE1b activates autophagy upon ER stress through Regulated Ire1-Dependent Decay of Messenger RNA (RIDD) activity, which is the degradation of mRNA by IRE1 upon ER stress. Three genes of which the mRNA are degraded by IRE1b were identified as negative regulators of autophagy upon ER stress. I performed the transient expression experiments for assessing autophagy in IRE1 mutants shown in figure 2 and in mutants of RIDD pathway candidate genes shown in figure 5. Xiang Yu performed the RNA-seq analysis. Yan Bao performed the remaining experiments. Diane C. Bassham and Stephen H. Howell wrote the manuscript. I also helped editing the manuscript, and my total contribution to this chapter is about 30%.

Chapter 5 is a general conclusion and discussion of this research with proposed future work. My contribution to this chapter is 100%.

CHAPTER 2

TOR-DEPENDENT AND –INDEPENDENT PATHWAYS REGULATE AUTOPHAGY IN ARABIDOPSIS

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2.1 Abstract

Autophagy is a critical process for recycling of cytoplasmic materials during environmental stress, senescence and cellular remodeling. It is upregulated under a wide range of abiotic stress conditions and is important for stress tolerance. Autophagy is repressed by the protein kinase target of rapamycin (TOR), which is activated in response to nutrients and in turn upregulates cell growth and translation and inhibits autophagy. Down-regulation of TOR in *Arabidopsis thaliana* leads to constitutive autophagy and to decreased growth, but the relationship to stress conditions is unclear. Here we assess the extent to which TOR controls autophagy activation under abiotic stress. Overexpression of *TOR* inhibited autophagy upon nutrient starvation, salt and osmotic stress, indicating that activation of autophagy under these conditions requires downregulation of TOR activity. In

contrast, TOR overexpression had no effect on autophagy induced by oxidative stress or ER stress, suggesting that activation of autophagy by these conditions is independent of TOR activity. The plant hormone auxin has been shown previously to up-regulate TOR activity. To confirm the existence of two pathways for activation of autophagy dependent on the stress conditions, auxin was added exogenously to activate TOR, and the effect on autophagy under different conditions was assessed. Consistent with the effect of TOR overexpression, the addition of the auxin NAA inhibited autophagy during nutrient deficiency, salt and osmotic stress, but not during oxidative or ER stress. NAA treatment was unable to block autophagy induced by a TOR inhibitor or by a mutation in the TOR complex component *RAPTOR1B*, indicating that auxin is upstream of TOR in the regulation of autophagy. We conclude that repression of auxin-regulated TOR activity is required for autophagy activation in response to a subset of abiotic stress conditions.

2.2 Introduction

Plants have evolved many response mechanisms to adapt to various growth conditions, including abiotic stresses. One such mechanism is autophagy, a major pathway for degradation and recycling of cytoplasmic materials in all eukaryotes (Liu and Bassham 2012, Yang and Bassham 2015). Autophagy is active at a low basal level even under normal conditions, and numerous human diseases are linked to autophagy defects, including cancer and various neurodegenerative diseases such as Parkinson's, Huntington's and Alzheimer's diseases (Cai, Arikkath et al. 2016, Davidson and Heiden 2017). In plants, autophagy functions in the response to both abiotic and biotic stress, and is induced during senescence and nutrient deficiency (Doelling, Walker et al. 2002, Hanaoka, Noda et al. 2002), salt and

drought stresses (Liu, Xiong et al. 2009), oxidative stress (Xiong, Contento et al. 2007), endoplasmic reticulum (ER) stress (Liu, Burgos et al. 2012) and pathogen infection (Liu, Schiff et al. 2005).

When autophagy is activated, a double-membrane cup-shaped structure named a phagophore is formed. The phagophore expands to form a double-membrane vesicle called an autophagosome, while engulfing cellular components to be degraded. Autophagosomes are delivered to and fuse with lysosomes in mammalian cells or the vacuole in plant or yeast cells, where the cargo is degraded into small molecules by vacuolar hydrolases and recycled (Yang and Bassham 2015). Studies in yeast have identified more than 30 autophagy-related (*ATG*) genes, many of which have also been found in plants (Tsukada and Ohsumi 1993, Yang and Bassham 2015). A key protein involved in autophagosome formation is ATG8, which can be used as a marker for autophagosomes when fused with a fluorescent protein (Yoshimoto, Hanaoka et al. 2004, Contento, Xiong et al. 2005). ATG8 is attached to the autophagosome membrane through a covalent bond to phosphatidylethanolamine (PE) via two ubiquitin-like conjugation systems that include the E1-like activating enzyme ATG7 (Ichimura, Kirisako et al. 2000). Knockout of *ATG7* therefore prevents autophagosome formation, leading to plants being hypersensitive to both abiotic and biotic stress conditions (Doelling, Walker et al. 2002, Lenz, Haller et al. 2011, Zhou, Wang et al. 2013).

The target of rapamycin (TOR) complex is a key regulator of autophagy, and is composed of TOR itself and two binding partners, regulatory-associated protein of TOR (RAPTOR), and Lethal with Sec Thirteen 8 (LST8) (Yang, Rudge et al. 2013, Dobrenel, Caldana et al. 2016). TOR is a Ser/Thr protein kinase in the phosphatidylinositol-3-kinase (PI3K) - related kinase (PIKK) family (Noda and Ohsumi 1998, Menand, Desnos et al.

2002), whereas RAPTOR recruits substrates to the complex for phosphorylation by TOR (Hara, Maruki et al. 2002), and LST8 stabilizes the complex (Yang, Rudge et al. 2013). The TOR signaling pathway both positively regulates cell growth and metabolism and negatively regulates autophagy in yeast, mammals and plants (Dobrenel, Caldana et al. 2016). In *Arabidopsis thaliana*, a null mutation in *TOR* is embryo lethal (Menand, Desnos et al. 2002), whereas decreased *TOR* expression due to RNA interference leads to autophagy induction (Liu and Bassham 2010), and arrested plant growth and development (Deprost, Yao et al. 2007). Active-site TOR inhibitors (asTORis) that disrupt TOR activity by competition for ATP-binding also result in plant growth defects (Montane and Menand 2013). Consistent with this, overexpression of *TOR* enhances plant growth and osmotic stress resistance (Deprost, Yao et al. 2007, Ren, Qiu et al. 2011).

Two *RAPTOR* genes exist in Arabidopsis, *RAPTOR1A* and *RAPTOR1B* (Anderson, Veit et al. 2005, Deprost, Truong et al. 2005). A *raptor1b* null mutant has growth defects, including delayed leaf initiation and growth, later bolting and flowering, and short roots, while *raptor1a* knock out mutants have no major developmental phenotypes, possibly due to the higher expression of *RAPTOR1B* in most plant tissues (Anderson, Veit et al. 2005, Deprost, Truong et al. 2005). A *raptor1a raptor1b* double knockout mutant has minimal meristem growth, indicating that RAPTOR1A and RAPTOR1B might have some distinct functions, but is not embryo-lethal, and TOR must therefore retain some of its function in the absence of RAPTOR (Anderson, Veit et al. 2005). Two *LST8* genes have also been identified in Arabidopsis, *LST8-1* and *LST8-2*, although only *LST8-1* appears to be expressed (Moreau, Azzopardi et al. 2012). The null mutant *lst8-1* has strong growth defects and impaired adaptation to long day conditions (Moreau, Azzopardi et al. 2012). Mutation of *lst8-1* or

raptor1b, or disruption of TOR activity with asTORis, causes hypersensitivity to abscisic acid (ABA) and decreased ABA synthesis (Kravchenko, Citerne et al. 2015), indicating that the TOR complex may also play a role in hormone signaling.

TOR signals through phosphorylation of downstream substrates (Raught, Gingras et al. 2001, Ren, Qiu et al. 2011). Several TOR substrates have been identified in Arabidopsis, including the 40S ribosomal protein S6 ribosomal p70 S6 kinase (S6K) (Mahfouz, Kim et al. 2006, Xiong and Sheen 2012), the E2Fa transcription factor, which regulates cell proliferation in animals (Xiong, McCormack et al. 2013), and TAP46, a regulatory subunit of protein phosphatase type 2A (PP2A), which was suggested to regulate plant growth and autophagy (Yorimitsu, He et al. 2009, Ahn, Han et al. 2011). Arabidopsis has two S6K paralogs with 87% sequence identity, S6K1 and S6K2, both of which are phosphorylated by TOR. The activity of plant S6Ks increases in response to auxin and cytokinins (Turck, Zilbermann et al. 2004).

Upstream regulation of TOR signaling in plants is still poorly understood. A recent study revealed that auxin can enhance TOR activity to promote the translation reinitiation of mRNAs via S6K1, and deficiency in TOR signaling impaired auxin-mediated root gravitropism (Schepetilnikov, Dimitrova et al. 2013). These studies indicate that auxin might regulate plant growth, development and stress responses through the TOR signaling pathway. In this study, we first confirm that the TOR complex is a negative regulator of autophagy in Arabidopsis, and demonstrate a role for RAPTOR1B in this regulation. We show that TOR regulates autophagy induced by nutrient starvation, salt or osmotic stress, but not oxidative or ER stress, indicating that TOR-dependent and -independent pathways for regulation of autophagy exist in plants. In addition, exogenous auxin has similar effects on stress-induced

autophagy as TOR overexpression, suggesting a mechanism by which auxin interfaces with stress responses in plants through regulation of TOR activity.

2.3 Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana seeds of WT (Col-0) or other genotypes were sterilized with 33% (v/v) bleach and 0.1% (v/v) Triton X-100 (Sigma) for 20 min, followed by 5 washes of 5 minutes each with sterile water. Sterilized seeds were stored at 4°C in darkness for at least 2 days to allow stratification before plating on solid ½ MS medium (2.22g/L Murashige-Skoog vitamin and salt mixture [Caisson Laboratory, MSP09], 1% [w/v] sucrose, 0.6% [w/v] Phytoblend agar [Caisson Laboratory], 2.4 mM 2-morphinolino-ethanesulfonic acid [MES, Sigma], pH 5.7). Seedlings were grown under long-day conditions (16 h light) at 22 °C for 7 days. Plants for transient expression in leaf protoplasts were grown in soil in a humidity-controlled growth chamber with 50% humidity at 20-23 °C under long-day conditions for 4 to 6 weeks. T-DNA insertion mutants used in this study are: *raptor1a* (SALK_043920c), *raptor1b* (SALK_078159) (Anderson, Veit et al. 2005), S7817 (SALK_147817), G166 (GABI_166C06), G548 (GABI_548G07) (Deprost, Yao et al. 2007), and *atg7* (GABI_655B06) (Chung, Phillips et al. 2010). Transgenic plants used in this study are: GFP-ATG8e (Xiong, Contento et al. 2007), TOR-OE1 and TOR-OE2 (Ren, Qiu et al. 2011).

Stress and drug treatment

For sucrose and nitrogen starvation, 7-d-old seedlings grown on solid ½ MS medium were transferred to solid ½ MS medium lacking sucrose or nitrogen for an additional 3 days.

Sucrose starvation plates were kept in the dark after transfer. For salt and mannitol treatment, 7-d-old seedlings grown on solid ½ MS medium were transferred to liquid ½ MS medium with 0.16 M NaCl or 0.35 M mannitol for 6-8 hours. For oxidative and ER stress, 7-d-old seedlings grown on solid ½ MS medium were transferred to liquid ½ MS medium with 5 mM H_2O_2 (Sigma) for 2-3 hours, or with 2 mM dithiothreitol (DTT, Fisher) or 5 μg/mL Tunicamycin (Sigma) for 6-8 hours. For AZD8055 treatment, 7-d-old seedlings grown on solid ½ MS medium were transferred to solid ½ MS medium with 2 μM AZD8055 (LC Laboratories) for 1 day, or liquid ½ MS medium with 1 μM AZD8055 for 2-3 hours.

For auxin treatment, 7-d-old seedlings were transferred to solid ½ MS medium supplemented with 20 nM 1-Naphthaleneacetic acid (NAA, Sigma-Aldrich, N0640) with or without starvation for an additional 3 days, or in liquid ½ MS medium with 20 nM NAA for 6-8 hours with or without stress treatments as described above.

For concanamycin A treatment, 7-d-old GFP-ATG8e seedlings were transferred to liquid ½ MS medium with DMSO or 1 μM concanamycin A (Sigma) with or without other stress or drug treatments for 6-8 hours

Autophagy detection by fluorescence microscopy

Arabidopsis seedling roots were stained with monodansylcadaverine (MDC) as described previously (Contento, Xiong et al. 2005). MDC-stained seedlings were observed with a Zeiss Axio Imager.A2 upright microscope (Zeiss) equipped with Zeiss Axiocam BW/color digital cameras using a DAPI-specific filter at the Iowa State University Microscopy and Nanoimaging Facility. GFP-ATG8e transgenic seedlings were observed and photographed with the same microscope system with a GFP-specific filter. Cells within the

root elongation zone were photographed and the number of autophagosomes in each image was counted and averaged from at least 10 images per sample. Confocal microscopy images of autophagosomes in root cells and leaf protoplasts were taken using a Leica SP5 × MP confocal/multiphoton microscope system (Leica) with a 63x/1.4 oil immersion objective at the Iowa State University Roy J. Carver High Resolution Microscopy Facility (Pu and Bassham 2016).

Transient expression in protoplasts

GFP-ATG8e was transiently expressed in Arabidopsis leaf protoplasts as previously described (Sheen 2002, Liu, Burgos et al. 2012). 25-30 μg of GFP-ATG8e plasmid DNA was introduced into protoplasts using 40% (w/v) polyethylene glycol (PEG, Sigma-Aldrich). Protoplasts were washed and incubated in W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES, pH 5.7). For starvation treatment, protoplasts were incubated in W5 solution without sucrose or with 0.5% (w/v) sucrose as control at room temperature in darkness for 2 days in total. For other stress treatments, protoplasts were incubated in W5 solution with treatments as described in the Stress and Auxin Treatment section. Protoplasts were observed by fluorescence microscopy (Nikon Eclipse E200) using a FITC filter, and protoplasts with more than 3 visible autophagosomes were counted as active for autophagy (Yang, Srivastava et al. 2016). A total of 100 protoplasts were observed per genotype for each condition, and the percentage of protoplasts with induced autophagy was calculated and averaged from 3 independent replicates.

Generation of RAPTOR1B construct

The *RAPTOR1B* cDNA sequence was divided into 2 fragments, and each fragment was amplified from Col-0 cDNA using CloneAmp HiFi PCR Premix (Takara). The 5' fragment of *RAPTOR1B* was amplified with forward primer 5'-CACCGAGCTCGAATTCATGGCATTAGGAGACTTAATGGTGTCTC-3' (inserted SacI restriction site underlined), and reverse primer 5'-GTCAAACCCAATATCAAGCAAGGTACCCA-3', digested with SacI and KpnI (within the *RAPTOR1B* cDNA sequence), and ligated into the pPZP212 binary vector (Hajdukiewicz, Svab et al. 1994, Li, Yu et al. 2009), which has a 35S promoter sequence at the 5' end, and a MYC tag sequence at the 3' end of the insert. The 3' fragment was amplified with forward primer 5'- TGGGTACCTTGCTTGATATTGGGTTTGAC-3' and reverse primer 5'- CACCGTCGACTCTTGCTTGCGAGTTGTCGTGGGTG-3' (inserted SalI restriction site underlined), digested with KpnI and SalI, and ligated into the pPZP212 vector containing the 5' fragment to complete the full sequence. The entire construct was confirmed by sequencing.

Accession numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative under the following accession numbers: TOR, AT1G50030; RAPTOR1A, AT5G01770, RAPTOR1B, AT3G08850; ATG8e, AT2G45170; ATG7, AT5G45900.

2.4 Results

2.4.1 Inhibition of TOR signaling leads to constitutive autophagy

We have shown previously that decreased *TOR* expression via RNA interference induces autophagy in Arabidopsis, suggesting that TOR is a negative regulator of autophagy in plants (Liu and Bassham 2010). To confirm that autophagy is induced by inhibition of TOR kinase activity (Montane and Menand 2013), we examined autophagy induction after application of the asTORis AZD8055 (Dong, Xiong et al. 2015). WT and *atg7* seedlings, a previously characterized knockout mutant that fails to form autophagosomes (Doelling, Walker et al. 2002), were grown under standard conditions for 7 days, followed by 1 μM AZD8055 treatment in liquid ½ MS medium for 2-3 hours. Roots of seedlings were stained with monodansylcadaverine (MDC), an acidotropic dye that can stain autophagosomes (Biederbick, Kern et al. 1995, Contento, Xiong et al. 2005), and examined by fluorescence microscopy. Autophagosomes appear as rapidly moving fluorescent puncta, and the number of visible puncta in each image were counted for quantification. As expected, compared to the basal level of autophagy in the control, inhibition of TOR activity by AZD8055 led to a significant increase in the number of autophagosomes (Figure 1A), while no autophagosomes were detected in the *atg7* mutant. This confirmed that TOR negatively regulates autophagy in *Arabidopsis*, and that the kinase activity of TOR is critical for this regulation.

Previous studies have shown that down regulation of *TOR* or its binding partners *RAPTOR* and *LST8* leads to defects in plant growth and development (Anderson, Veit et al. 2005, Deprost, Yao et al. 2007, Moreau, Azzopardi et al. 2012, Montane and Menand 2013), suggesting that RAPTOR and LST8 are critical for TOR-regulated plant growth. To test whether inhibition of TOR complex activity by disruption of *RAPTOR* also induces

autophagy, WT, *raptor1a* and *raptor1b* knockout mutant seedlings (Anderson, Veit et al. 2005) were grown on ½ MS medium with sucrose for a week, and autophagy in root cells was examined by MDC staining followed by fluorescence microscopy (Figure 1B, C). Compared to the basal level of autophagy in WT seedlings, the number of autophagosomes in the *raptor1a* mutant appeared slightly higher, but this difference was not statistically significant, possibly due to the variability between seedlings. The *raptor1b* mutant had a significantly higher number of autophagosomes, suggesting that the *raptor1b* mutant has constitutive autophagy, and that *RAPTOR1A* and *RAPTOR1B* may not function equally in autophagy regulation.

To confirm that the *raptor1b* mutant has increased basal autophagy under standard conditions, the autophagosome marker GFP-ATG8e was expressed transiently in WT, *raptor1a* and *raptor1b* leaf protoplasts (Figure 1D). The GFP-ATG8e fusion protein has been used extensively as a specific marker of autophagosomes and autophagic bodies (Yoshimoto, Hanaoka et al. 2004, Contento, Xiong et al. 2005, Pu and Bassham 2016), and active autophagy is defined as more than 3 visible autophagosomes in a protoplast (Yang, Srivastava et al. 2016). WT protoplasts maintain a basal level of autophagy with a low percentage with active autophagy. Consistent with the MDC staining results, the percentage of *raptor1b* protoplasts with active autophagy was significantly higher than that of WT protoplasts (Figure 1E). However, *raptor1a* also had a significantly higher percentage of active autophagy in leaf protoplasts, although significantly lower than *raptor1b*. *RAPTOR1A* may therefore be more important for autophagy regulation in leaves than in roots.

To confirm that the constitutive autophagy in the *raptor1b* mutant is specifically due to the mutation in *RAPTOR1B*, the *RAPTOR1B* cDNA was transiently expressed under a 35S

promoter together with GFP-ATG8e in *raptor1b* knock out mutant leaf protoplasts. Autophagy was assessed as described above using fluorescence microscopy (Figure 1E). The percentage of protoplasts with active autophagy in the *raptor1b* mutant expressing the *RAPTOR1B* cDNA was substantially lower than for the mutant protoplasts alone, and was not significantly different to WT. This indicates that the increased basal autophagy observed in the *raptor1b* mutant was suppressed by expression of the *RAPTOR1B* cDNA, confirming that the constitutive autophagy phenotype is indeed due to the disruption of *RAPTOR1B*.

2.4.2 Overexpression of TOR blocks autophagy upon starvation, salt and drought stress

Previous studies and our data have shown that the TOR complex negatively regulates autophagy in Arabidopsis (Liu and Bassham 2010) (Figure 1), but the conditions under which TOR is important in plants are unknown. In other organisms TOR is well-described as regulating autophagy in response to nutrients (Dobrenel, Caldana et al. 2016), with a decrease in TOR activity during nutrient deficiency leading to activation of autophagy. We therefore hypothesized that overexpression of TOR might prevent activation of autophagy by nutrient deficiency, and that autophagy induction by other stresses might be TOR-independent. To test this hypothesis, we obtained several Arabidopsis lines with T-DNA insertions in the TOR upstream region (S7817, G166 and G548) and two transgenic lines with TOR expressed from a 35S promotor (TOR-OE1 and TOR-OE2). All lines have overexpression of TOR and enhanced growth (Deprost, Yao et al. 2007, Ren, Qiu et al. 2011), with the exception of S7817, which has decreased TOR expression in leaves and overexpression of TOR in roots (Deprost, Yao et al. 2007). Seeds of WT and the five TOR overexpression lines were germinated and grown on solid ½ MS medium plus sucrose for

one week, followed by transfer to solid ½ MS medium plus or minus nitrogen in the light, or minus sucrose in the dark for an additional 3 days. Autophagosomes were detected by MDC staining followed by fluorescence microscopy (Figure 2A, B). Representative images of one of the TOR overexpression lines are shown in Figure 2A. Quantification of autophagosomes indicated that both WT and the TOR overexpression lines had a low basal level of autophagy under control conditions. The average number of autophagosomes in WT seedlings after sucrose or nitrogen starvation was significantly higher than in control conditions, whereas the TOR overexpression lines had no significant activation of autophagy. This indicates that overexpression of TOR can repress autophagy induced by nutrient starvation, suggesting that repression of TOR activity is required for activation of autophagy in response to nutrient depletion.

While previous studies have shown that TOR is involved in nutrient sensing (Dobrenel, Caldana et al. 2016), the extent to which TOR regulates stress responses other than nutrient deficiency is not known, although a link to osmotic stress resistance in Arabidopsis has been suggested (Mahfouz, Kim et al. 2006, Deprost, Yao et al. 2007). Autophagy is activated in Arabidopsis by salt and osmotic stresses (Liu, Xiong et al. 2009). Therefore, we also tested whether overexpression of TOR affects autophagy induced by salt or osmotic stress (Figure 2A, C). WT and the TOR overexpression lines were germinated and grown on solid ½ MS medium for one week, and then transferred to liquid ½ MS medium containing 0.16 M NaCl or 0.35 M mannitol for 6 to 8 hours. Autophagy in seedling roots was assayed by MDC staining followed by fluorescence microscopy (Figure 2C). Autophagy in salt or mannitol treated WT seedlings was significantly higher than the basal level of autophagy seen under control conditions. Surprisingly, as for nutrient deficiency, autophagy

was not induced in TOR overexpression lines under salt or osmotic stress, indicating that TOR can also repress autophagy induced by these stresses.

To confirm these results, we measured autophagy by transient expression of GFP-ATG8e in leaf protoplasts from WT and TOR overexpressing plants under sucrose starvation, salt and osmotic stresses (Figure 2D and 2E). As the protoplast incubation buffer contains nitrogen, it was not possible to test nitrogen deficiency using our standard protocol. After transformation with GFP-ATG8e constructs, protoplasts were incubated with or without sucrose for 2 days (Figure 2D), or plus or minus 0.16 M NaCl or 0.35 M mannitol for 1 day (Figure 2E), after which autophagy was observed using fluorescence microscopy. The percentage of protoplasts with active autophagy was calculated, with 100 protoplasts observed per genotype for each condition. WT and TOR overexpression lines had a low level of autophagy under control conditions, except for the S7817 line which had constitutive activation of autophagy. In this line, TOR expression is decreased in leaves, potentially explaining this observation (Deprost, Yao et al. 2007). While WT protoplasts had a significantly higher level of autophagy under sucrose starvation, salt and osmotic stresses, autophagy in the TOR overexpression lines, with the exception of S7817, remained at a low basal level indistinguishable from that in control conditions. We conclude that TOR is a regulator of autophagy in response to salt and osmotic stress in addition to nutrient deficiency.

2.4.3 Overexpression of TOR has no effect on oxidative stress- or ER stress-induced autophagy

Autophagy is also induced by oxidative stress and ER stress in plants (Xiong, Contento et al. 2007, Liu, Burgos et al. 2012, Yang, Srivastava et al. 2016). Oxidative stress is triggered when cells accumulating excessive reactive oxygen species (ROS), and oxidized proteins are degraded through autophagy (Xiong, Contento et al. 2007). ER stress is generated when unfolded or misfolded proteins exceed the capacity of protein folding or degradation system, causing accumulation of proteins in the ER (Howell 2013). It can be triggered by salt, drought, and heat stresses, or experimentally by chemicals such as dithiothreitol (DTT) or tunicamycin (Howell 2013). To determine whether TOR regulates autophagy upon oxidative or ER stress, one-week-old WT and TOR overexpression lines were transferred to liquid ½ MS medium plus or minus 5 mM H_2O_2 for 2 to 3 hours to induce oxidative stress, or plus 2 mM DTT or 5 μg/mL tunicamycin for 6 to 8 hours to induce ER stress. Autophagy in seedling roots was detected by MDC staining followed by fluorescence microscopy (Figure 3A-C). Representative images of one of the TOR overexpression lines are shown in Figure 3A. WT and TOR overexpression lines had a low level of autophagy under control conditions, and WT seedlings has significantly higher autophagy induction after oxidative or ER stress treatment. Unlike nutrient, salt or osmotic stresses, TOR overexpression has no effect on autophagy induction, as overexpression lines remain able to activate autophagy under these stresses, suggesting that autophagy is activated via a pathway that does not require inhibition of TOR activity.

To confirm that autophagy remains induced in TOR overexpression lines under oxidative and ER stress, GFP-ATG8e was transiently expressed in leaf protoplasts of WT

and TOR overexpression lines. Protoplasts were incubated with or without 5 mM H_2O_2, 2 mM DTT or 5 μg/mL tunicamycin for one day, and observed using fluorescence microscopy (Figure 3D and 3E). WT protoplasts had a significantly higher level of autophagy after oxidative or ER stress treatment. In accordance with the MDC staining results, TOR overexpression lines also had significantly higher percentage of active autophagy after oxidative or ER stress treatment, with no significant difference compared to WT under the same stress conditions. This demonstrates that overexpression of TOR is unable to repress autophagy induced by oxidative or ER stress, suggesting that oxidative- or ER stress-induced autophagy might be regulated through a TOR-independent pathway.

2.4.4 Auxin represses stress-induced autophagy through TOR

TOR activity in Arabidopsis can be enhanced by exogenous addition of the auxin 1-Naphthaleneacetic acid (NAA), (Schepetilnikov, Dimitrova et al. 2013), indicating that auxin might regulate plant growth and development via the TOR signaling pathway. We hypothesized that auxin might repress stress-induced autophagy in plants through the TOR pathway. To confirm the existence of TOR-dependent and -independent pathways for activation of autophagy, NAA was added exogenously to GFP-ATG8e transgenic plants to activate TOR, and the effect on autophagy under different conditions was assessed (Figure 4A-D). For nutrient deficiency, 7-d-old GFP-ATG8e transgenic seedlings were transferred to solid ½ MS medium with or without sucrose or nitrogen and plus 20 nM NAA or DMSO for an additional 3 days. For salt, osmotic and ER stress, 7-d-old seedlings were transferred to liquid ½ MS medium plus 0.16 M NaCl, 0.35 M mannitol, 2 mM DTT or 5 μg/mL tunicamycin and plus 20 nM NAA or DMSO for 6 to 8 hours. For oxidative stress, 7-d-old

seedlings were transferred to liquid ½ MS medium plus 20 nM NAA or DMSO for 6 to 8 hours, with 5 mM H_2O_2 added only during the last 2-3 hours to avoid cell death. To more clearly observe GFP-ATG8e-labeled autophagic bodies in the vacuoles by confocal microscopy, 1 μM concanamycin A was added to block degradation of autophagic bodies prior to imaging of the vacuoles (Dröse, Bindseil et al. 1993, Liu and Bassham 2010) (Figure 4A). In control conditions, root cells had few autophagic bodies within the vacuole, whereas all stresses tested led to accumulation of large numbers of autophagic bodies. In the presence of auxin, autophagic body accumulation was inhibited in nutrient deficiency, salt and osmotic stress, but accumulation was still observed in oxidative and ER stress. These results also indicate that NAA reduces the number of autophagosomes observed by blocking autophagosome formation, rather than by accelerating autophagosome degradation. Autophagy was quantified by counting the number of autophagic bodies in each frame, averaged from 10 images per genotype for each condition (Figure 4B-D). Compared to the basal level of autophagy under control conditions, autophagy was significantly higher after stress treatments. In the presence of NAA, autophagy was still significantly induced by oxidative and ER stress conditions, but no significant difference compared to control conditions was observed under nutrient starvation, salt and osmotic stresses. This suggests that NAA represses autophagy induced by sucrose and nitrogen starvation, salt and osmotic stresses, but not oxidative stress or ER stress, consistent with the results from overexpression of TOR.

To further confirm that addition of auxin represses stress-induced autophagy through activation of TOR, we examined whether auxin can inhibit the constitutive autophagy activated upon disruption of the TOR signaling pathway by chemical inhibition or genetic

mutation (Figure 4E-G). To inhibit TOR kinase activity, 7-d-old GFP-ATG8e seedlings were transferred to liquid ½ MS medium with or without 20 nM NAA for 6 to 8 hours, with DMSO or 1 μM AZD8055 added during the last 2 to 3 hours of treatment. GFP-labeled autophagic bodies in roots after concanamycin A treatment were examined using confocal microscopy (Figure 4A). AZD8055 as expected led to a high accumulation of autophagic bodies in the vacuole, and NAA had no effect on this accumulation, suggesting that NAA acts upstream of TOR in the autophagy pathway. The extent of autophagy was quantified by counting root autophagosomes, and AZD8055 caused accumulation of autophagosomes both in the presence and absence of NAA, with no significant difference in autophagy induction (Figure 4E).

As an alternative approach, the effect of NAA upon inhibition of TOR complex function via knockout of *RAPTOR1B* was tested. Seven-d-old WT and *raptor1b* seedlings were transferred to liquid ½ MS medium with or without 20 nM NAA for 6 to 8 hours, followed by MDC staining and autophagy detection by fluorescence microscopy (Figure 4F, G). NAA had no significant effect on the constitutive autophagy seen in the *raptor1b* mutant. Taken together, these results suggest that auxin acts upstream of TOR in the regulation of autophagy.

2.5 Discussion

The TOR signaling pathway is a complex and critical pathway for balancing cell growth and survival (Dobrenel, Caldana et al. 2016). TOR was suggested to function as a complex with RAPTOR and LST8 based on studies in yeast and mammals (González and Hall 2017), and previous studies in plants (Anderson, Veit et al. 2005, Deprost, Truong et al.

2005, Moreau, Azzopardi et al. 2012). Knock out mutation in TOR is embryo-lethal in Arabidopsis (Menand, Desnos et al. 2002). Down-regulation of TOR via RNA-interference arrests plant growth and induces autophagy, suggesting that TOR is a positive regulator of plant growth and development, and a negative regulator of autophagy in plants (Deprost, Yao et al. 2007, Liu and Bassham 2010). To confirm that the TOR complex regulates autophagy in Arabidopsis, we used a TOR inhibitor, AZD8055 (Chresta, Davies et al. 2010, Montane and Menand 2013, Dong, Xiong et al. 2015), which leads to a significant induction of autophagy (Figure 1A). This further confirmed that TOR is a negative regulator of autophagy in Arabidopsis. We also disrupted the TOR signaling pathway by knocking out *RAPTOR*, the binding partner of TOR. *RAPTOR1B* has been shown to be the most highly expressed isoform of RAPTOR in Arabidopsis (Deprost, Truong et al. 2005), and *raptor1b* has a much more severe growth defect than *raptor1a* (Anderson, Veit et al. 2005). The *raptor1b* knockout line exhibits constitutive autophagy in both roots and leaf protoplasts, and significantly severer than *raptor1a* mutant, suggesting disruption of the TOR binding partner RAPTOR also leads to autophagy induction. Taken together, we confirmed that both TOR and RAPTOR function as a negative regulator of autophagy in Arabidopsis, and *RAPTOR1B* might be more critical for autophagy regulation than *RAPTOR1A*.

Autophagy is induced by numerous stresses, including nutrient deficiency, salt, drought, oxidative and ER stresses (Doelling, Walker et al. 2002, Hanaoka, Noda et al. 2002, Xiong, Contento et al. 2007, Liu, Xiong et al. 2009, Liu, Burgos et al. 2012). TOR has been well characterized as regulating autophagy in response to nutrients in yeast and mammals, and down-regulation of TOR leads to growth defects and autophagy induction (Dobrenel, Caldana et al. 2016). Therefore, we hypothesized that nutrient deficiency induces autophagy

through the TOR signaling pathway in plants. As expected, overexpression of *TOR* repressed autophagy induced by sucrose or nitrogen starvation, suggesting TOR regulates nutrient deficiency induced autophagy. Many upstream regulator of TOR have been found in yeast and mammals, although many of them have not been identified in plants. One of the conserved upstream regulator of TOR, AMPK in mammals and Snf1 in yeast, were shown to be critical nutrient and energy sensors for autophagy regulation. The homolog of AMPK and Snf1 in plants, SnRK1, has been identified, which is also activated under stress conditions (Dobrenel, Caldana et al. 2016). However, the interaction of SnRK1 regulation and TOR signaling pathway in plants is still under studied. Further studies are needed to test whether SnRK1 regulates TOR in response to nutrient deficiency and other stress conditions.

Salt and drought stresses are two major environmental stresses encountered by plants; both lead to osmotic stress, while salt stress also leads to ionic stress. Surprisingly, overexpression of TOR and activation of TOR by auxin represses autophagy in both conditions, indicating that activation of autophagy upon salt and drought stress is also dependent on TOR. A substrate of TOR, S6K, shows reduced expression and activity under salt and osmotic stress (Mizoguchi, Hayashida et al. 1995, Mahfouz, Kim et al. 2006), suggesting salt and osmotic stress reduce TOR activity. However, it is unclear how the TOR signaling pathway receives salt and osmotic stress signals for regulation of autophagy. Salt, osmotic stress and nutrient deficiency increase cellular ROS levels, which might function as signaling molecules, or leads to oxidative stress (Zhu 2016). One of the major source of signaling ROS is generated by plasma membrane NADPH oxidases (Miller, Schlauch et al. 2009). Previous study showed that application of the NADPH oxidase inhibitors inhibits autophagy induced by nutrient deficiency and salt stress, but not osmotic stress (Liu, Xiong

et al. 2009), suggesting that autophagy activated by osmotic stress is independent of NADPH oxidase. NADPH oxidase inhibitors also failed to inhibit autophagy induced by down regulation of *TOR* by RNA interference (Liu and Bassham 2010). These results suggest that TOR maybe downstream of NADPH oxidase, or in a parallel pathway that is independent of NADPH oxidase in activation of autophagy. However, excessive ROS trigger oxidative stress, and up-regulation of TOR by overexpression or auxin failed to repress autophagy induced by H_2O_2, suggesting oxidative stress activates autophagy through a TOR-independent pathway. It is still unclear whether signaling ROS regulates autophagy through TOR, and future investigation are needed to characterize different stress sensors in regulation of autophagy.

Salt, drought and heat stresses also might cause accumulation of excessive unfolded or misfolded proteins within cells, which leads to ER stress. ER stress has been shown to induce autophagy in Arabidopsis (Liu, Burgos et al. 2012, Yang, Srivastava et al. 2016). However, our data indicates that TOR overexpression has no effect on ER stress, suggesting that ER stress-induced autophagy is independent of TOR. Upon ER stress, the plant ER stress sensor inositol-requiring enzyme-1 (IRE1) splices the mRNA encoding the transcription factor membrane-associated basic leucine zipper 60 (bZIP60) to activate the unfolded protein response (UPR). The UPR aids proper folding or degradation of unfolded and misfolded proteins via upregulation of UPR-related genes (Howell 2013). In Arabidopsis, induction of autophagy by ER stress is dependent on one of the IRE1 isoforms, IRE1b, but not IRE1a or bZIP60 (Liu, Burgos et al. 2012). Our recent research showed that unfolded and misfolded proteins activate autophagy in an IRE1b dependent pathway (Yang, Srivastava et al. 2016). Taken together with our results, ER stress regulated autophagy might through the IRE1b

pathway, and is independent of TOR. However, how IRE1b regulates autophagy upon ER stress, and whether other UPR response genes are involved in regulation of autophagy requires further investigation.

Auxin has long been studied for its critical role in plant growth regulation (Enders and Strader 2015). Auxin has been shown to enhance TOR activity, and auxin-mediated root gravitropism is impaired when TOR signaling is disrupted (Schepetilnikov, Dimitrova et al. 2013). Auxin also failed to restore hypocotyl growth in estradiol-inducible *tor* mutants (Zhang, Zhu et al. 2016), and many auxin response genes have reduced expression upon inhibition of TOR (Dong, Xiong et al. 2015), suggesting that TOR is involved in auxin-regulated plant growth. Recent studies identified a small GTPase, ROP2, mediates the activation of TOR by auxin (Li, Cai et al. 2017, Schepetilnikov, Makarian et al. 2017), which further characterized how auxin activates TOR activity. Therefore, we hypothesized that enhancing TOR activity by auxin might also repress stress-induced autophagy regulated through the TOR signaling pathway. Indeed, same as in the TOR overexpression lines, autophagy induced by nutrient starvation, salt and osmotic stresses were repressed by addition of NAA, whereas oxidative and ER stresses induced autophagy was not affected. By contrast, NAA was unable to repress the autophagy induced by disruption of TOR activity with the inhibitor AZD8055 or in a *raptor1b* knockout line. Exogenous application of the synthetic auxin 2, 4-D failed to restore growth of *raptor1b*, although *raptor1b* mutants can sense exogenous auxin normally (Anderson, Veit et al. 2005), which further supports the conclusion that TOR signaling acts downstream of auxin. In summary, we confirmed that autophagy induced by abiotic stresses are regulated through TOR-dependent or –independent pathways, and auxin regulates plant stress responses through the TOR signaling pathway.

Future work is required to identify the upstream stress sensors that repress TOR activity to allow activation of autophagy, and components of the TOR-independent autophagy activation pathway.

2.6 Acknowledgments

We thank Dr. Maureen Hanson for providing *raptor1a* and *raptor1b* mutant seeds, Dr. Raju Datla for TOR-OE1 and TOR OE2 mutant seeds, Dr. Richard Vierstra for *atg7-2* mutant seeds, and Dr. Yanhai Yin for the pPZP212 vector. We thank Dr. Margaret Carter for her assistance with confocal microscopy, and Dr. Harry Horner, Dr. Randall Den Adel and Dr. Tracey Stewart for their assistance with epifluorescence microscopy.

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2.8 Figures and tables

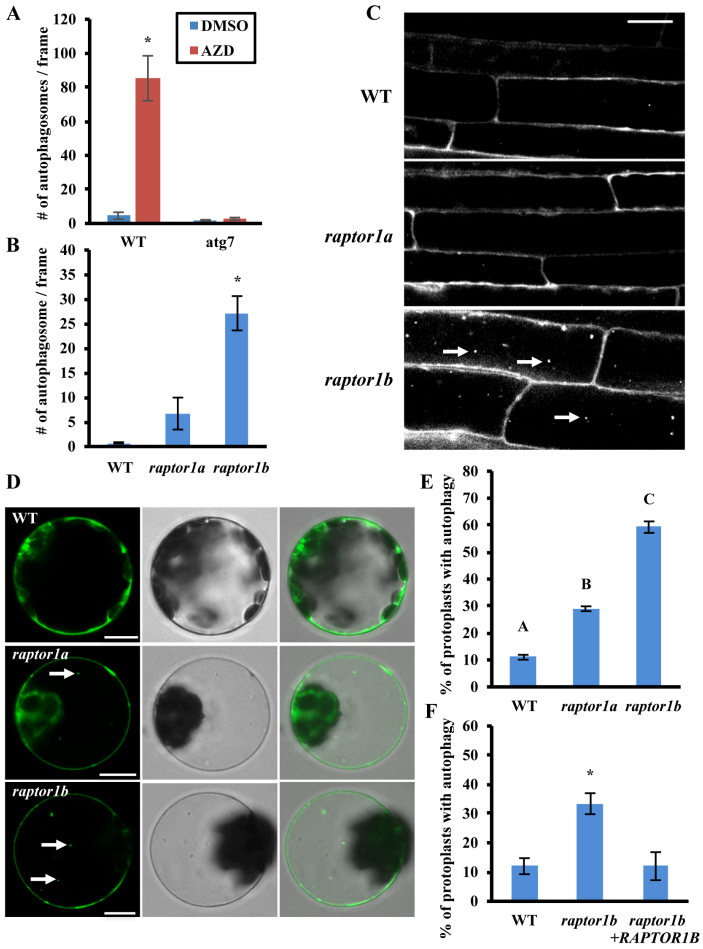

Figure 1. Inhibition of TOR or RAPTOR leads to constitutive autophagy.

(A) The TOR inhibitor AZD8055 induces autophagy. 7-day-old WT (Col-0) and *atg7* mutant seedlings were treated with DMSO or AZD8055 (AZD) for 2-3 hours, stained with MDC and then observed and imaged by fluorescence microscopy. The number of puncta in each image was counted and averaged from at least 10 images per genotype for each condition. **(B, C)** Autophagy is induced in a *raptor1b* mutant root cells under standard growth conditions. (**B)** 7-day-old WT, *raptor1a* and *raptor1b* knockout mutant seedlings were stained with MDC and observed by fluorescence microscopy. The number of puncta in each image was quantified as in A. **(C)** Representative confocal images of MDC-stained WT, *raptor1a* and *raptor1b* mutant seedlings. MDC-stained autophagosomes appear as white puncta within cells as indicated by white arrows. Scale bar = 20 μm. **(D, E)** *raptor1a* and *raptor1b* mutants have constitutive autophagy in leaf protoplasts. **(D)** Transient expression of a GFP-ATG8e fusion protein in leaf protoplasts of WT and *RAPTOR* mutants, observed by confocal microscopy. GFP-tagged autophagosomes appear as green puncta within leaf protoplasts in the left column as indicated by white arrows. The middle and right columns show DIC and merged images respectively. Scale bar = 10 μm. **(E)** Quantification of D. Protoplasts were observed using fluorescence microscopy. The percentage of protoplasts with more than 3

visible GFP-tagged autophagosomes was calculated, with 100 protoplasts observed per genotype for each condition. **(F)** Expression of the *RAPTOR1B* cDNA complements the *raptor1b* constitutive autophagy phenotype. A GFP-ATG8e fusion protein was transiently expressed in *raptor1b* mutant leaf protoplasts with or without full-length *RAPTOR1B*, or in WT protoplasts as a control, expressed from a 35S constitutive promoter. Protoplasts were observed using fluorescence microscopy. The percentage of protoplasts with more than 3 visible GFP-tagged autophagosomes was quantified as in E. For all graphs, error bars indicate means ± standard error (SE) from three independent replicates. Asterisks or different letters indicate statistically significant differences ($P < 0.05$) using Student's *t*-test compared with WT under control conditions.

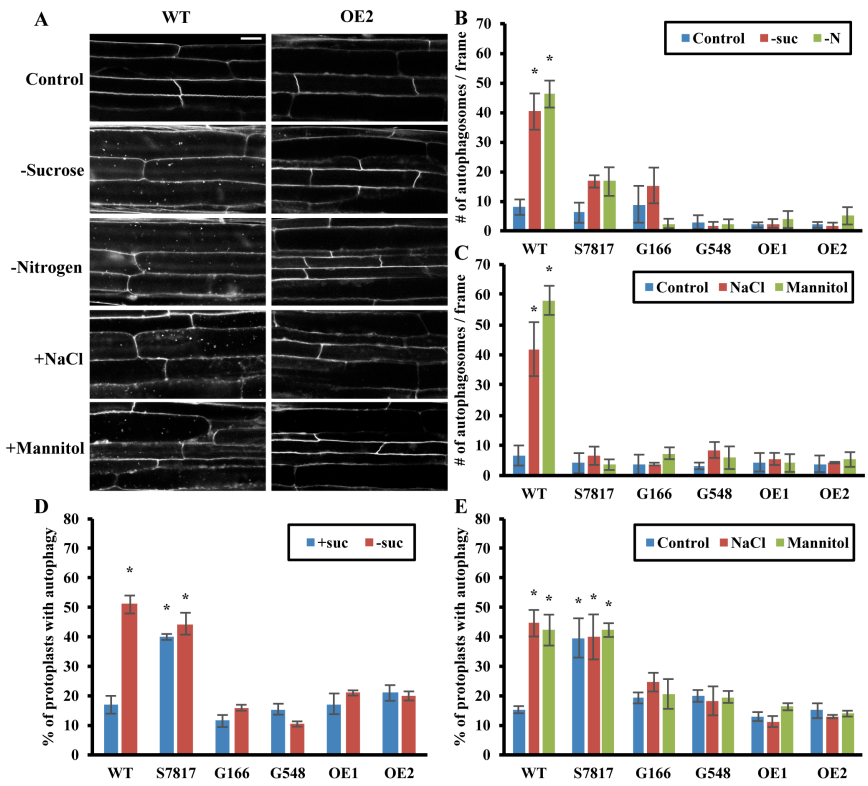

Figure 2. Overexpression of TOR blocks autophagy induced by nutrient starvation, salt and osmotic stresses.

(**A**) Representative confocal images of MDC-stained WT and TOR-OE2 seedlings after indicated stress treatment. For nutrient starvation, 7-day-old seedlings of WT and TOR-OE2 transgenic lines were transferred to solid ½ MS medium for an additional 3 days with or without nitrogen in the light, or without sucrose in the dark. For salt and osmotic stress, 7-day-old WT and TOR-OE2 seedlings were transferred to liquid ½ MS medium plus or minus 0.16 M NaCl or 0.35 M mannitol for 6-8 hours. Scale bar = 20 μm. **(B, C)** Quantification of autophagosome number in WT and TOR overexpression lines under sucrose or nitrogen starvation **(B)**, salt, or osmotic stress **(C)**, treated as in A. MDC-stained autophagosomes were observed by fluorescence microscopy and photographed. The average number of autophagosomes was calculated from 10 images per genotype for each condition. **(D, E)** TOR overexpression lines fail to activate autophagy under sucrose starvation **(D)**, salt or osmotic stress **(E)** in leaf protoplasts. The GFP-ATG8e fusion protein was transiently expressed in leaf protoplasts of WT and TOR overexpression lines. Protoplasts were incubated in the dark plus or minus 0.5% (w/v) sucrose for 2 days **(D)**, or plus or minus 0.16 M NaCl or 0.35 M mannitol for 1 day **(E)**. Protoplasts were observed using fluorescence microscopy. The percentage of protoplasts with more than 3 visible GFP-tagged autophagosomes was calculated from 100 protoplasts observed per genotype for each condition. For all graphs, error bars indicate means ± SE from three independent replicates. Asterisks indicate statistically significant differences ($P < 0.05$) using Student's *t*-test compared with WT under control conditions.

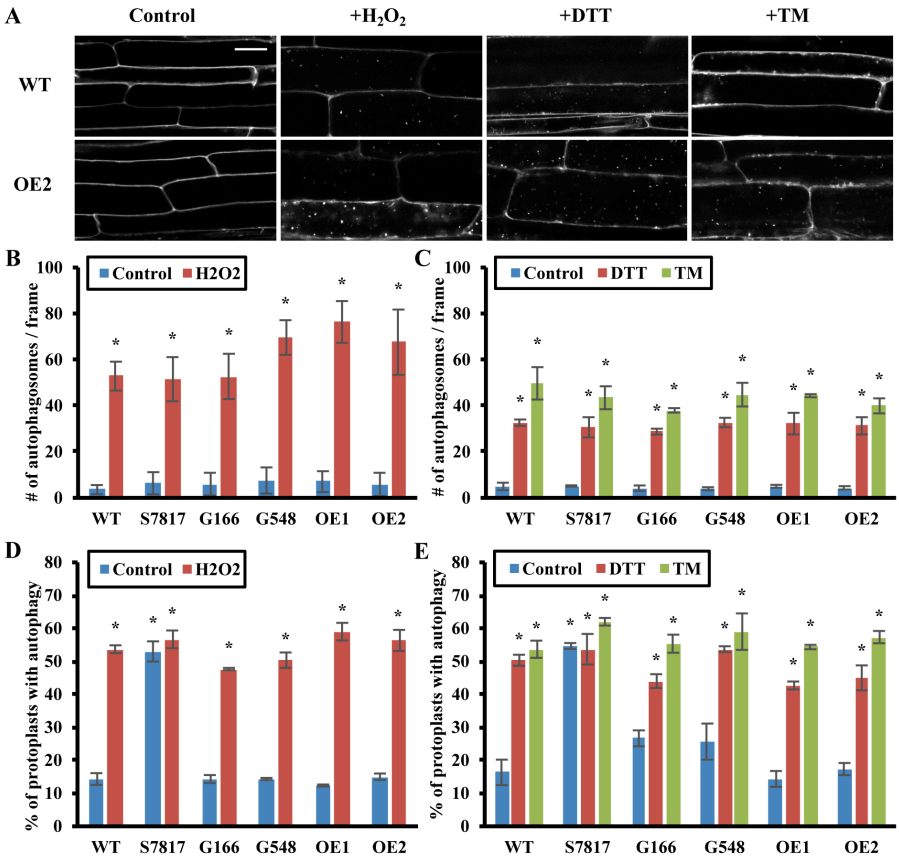

Figure 3. Overexpression of TOR has no effect on oxidative stress- or ER stress-induced autophagy.

(A) Confocal microscopy of MDC-stained WT and TOR-OE2 seedlings after the indicated stress treatment. 7-day-old WT and TOR overexpression transgenic seedlings were transferred to liquid ½ MS medium with or without 5 mM H_2O_2 for 2-3 hours for oxidative stress, or 2 mM DTT or 5 µg/mL tunicamycin (TM) for 6-8 hours for ER stress. Scale bar = 20 µm. **(B, C)** Quantification of autophagosome number in WT and TOR overexpression lines under oxidative stress **(B)** or ER stress **(C)** treated as in A. MDC-stained autophagosomes were observed by fluorescence microscopy and photographed. The average number of autophagosomes was calculated from 10 images per genotype for each condition. **(D, E)** Autophagy was induced in leaf protoplasts of TOR overexpression lines under oxidative stress **(D)** or ER stress **(E)**. AGFP-ATG8e fusion protein was transiently expressed in leaf protoplasts of WT and TOR overexpression lines. Protoplasts were incubated in the dark plus or minus 5 mM H_2O_2 for 2-3 hours **(D)**, or 2 mM DTT or 5 µg/mL tunicamycin (TM) for 6-8 hours **(E)**. Protoplasts were observed using fluorescence microscopy. The percentage of protoplasts with more than 3 visible GFP-tagged autophagosomes was calculated from 100 protoplasts observed per genotype for each condition. For all graphs, error bars indicate means ± SE from three independent replicates. Asterisks indicate statistically significant differences ($P < 0.05$) using Student's *t*-test compared with WT under control conditions.

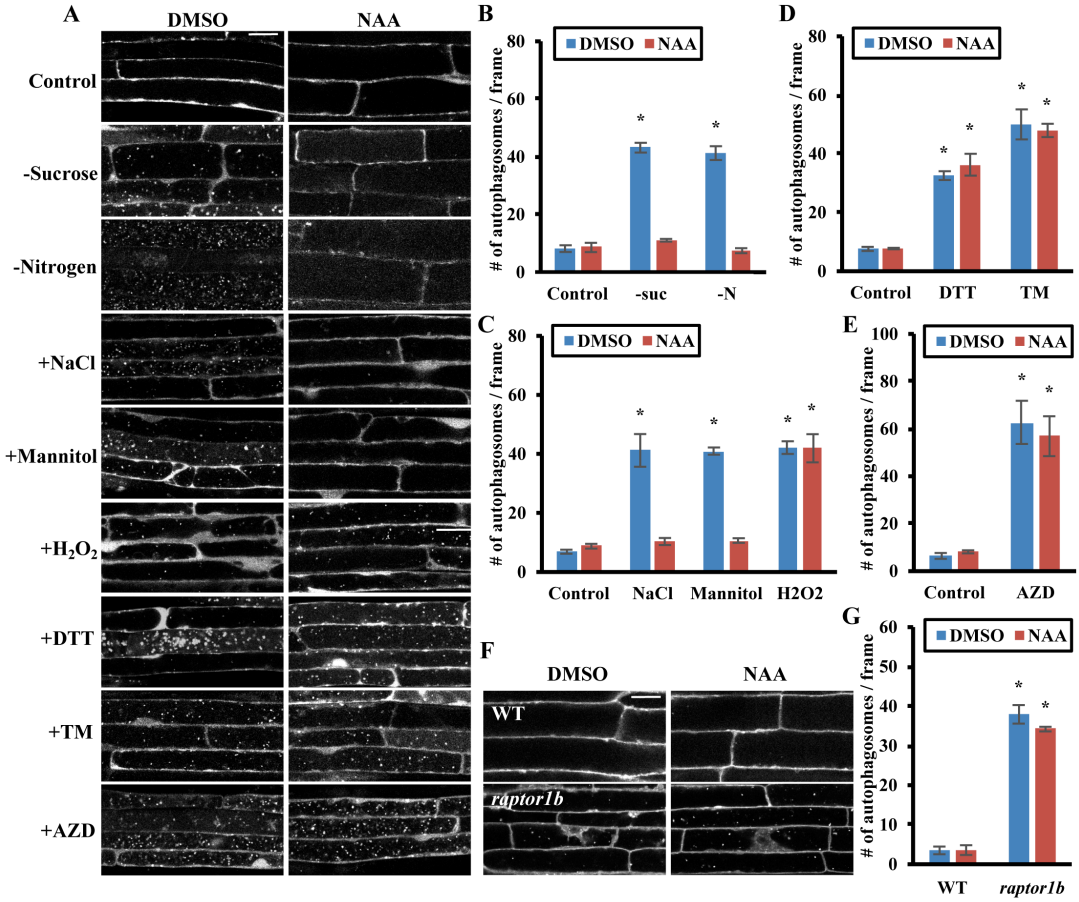

Figure 4. Auxin represses autophagy induced by nutrient starvation, salt and osmotic stress through the TOR signaling pathway.

(A-E) NAA represses autophagy induced by nutrient starvation, salt and osmotic stresses. **(A)** Representative confocal images of GFP-ATG8e transgenic seedlings after NAA and stress treatments. Concanamycin A was included under all conditions to allow accumulation of autophagic bodies inside the vacuole, facilitating visualization. For nutrient starvation, 7-day-old GFP-ATG8e seedlings were transferred to solid ½ MS medium plus DMSO or 20 nM NAA for an additional 3 days with or without nitrogen in the light, or without sucrose in the dark. Treated seedlings were then transferred to liquid medium under the same conditions plus 1 μM concanamycin A for an additional 6-8 hours. For all other stresses, 7-day-old GFP-ATG8e seedlings were transferred to liquid ½ MS medium with 1 μM concanamycin A and DMSO or 20 nM NAA for 6-8 hours, together with 0.16 M NaCl, 0.35 M Mannitol, 2 mM DTT, or 5 μg/mL tunicamycin (TM) for 6-8 hours, or 5 mM H_2O_2 or 1 μM AZD8055 during the last 2-3 of DMSO or NAA treatment. Scale bar = 20 μm. **(B-E)** Quantification of autophagic body number in GFP-ATG8e transgenic seedlings under sucrose or nitrogen starvation (B), salt, osmotic stress or oxidative stress **(C)**, ER stress **(D)**, or AZD8055 treatment **(E)**, treated as in A. GFP-tagged autophagosomes in each condition were observed by fluorescence microscopy and photographed. The number of autophagosomes was counted

and averaged from 10 images per genotype for each condition. **(F, G)** Auxin cannot repress the constitutive autophagy seen in *raptor1b* mutant. **(F)** Representative confocal images of MDC-stained WT and *raptor1b* mutant seedling roots. 7-day-old WT and *raptor1b* seedlings were treated in liquid ½ MS medium with DMSO or 20 nM NAA for 6-8 hours. Scale bar = 20 μm. **(G)** Quantification of F. The average number of autophagosomes was calculated from 10 images per genotype for each condition. For all graphs, error bars indicate means ± SE from three independent replicates. Asterisks indicate statistically significant differences ($P < 0.05$) using Student's *t*-test compared with WT under control conditions.

CHAPTER 3

BRASSINOSTEROIDS REGULATE PLANT GROWTH AND AUTOPHAGY THROUGH PHOSPHORYLATION OF TARGET OF RAPAMYCIN (TOR) BY BRASSINAZOLE-INSENSITIVE 2 (BIN2)

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3.1 Abstract

Brassinosteroids (BRs) are a family of plant hormones that are essential for regulation of plant growth and development. BRs signal through the negative regulator Brassinazole-Insensitive 2 (BIN2), a protein kinase that regulates transcription factor activity to control plant growth and stress responses. Target of Rapamycin (TOR) is an essential positive regulator of plant growth and a negative regulator of autophagy. In this study, we showed that BRs regulate plant growth and autophagy through BIN2 phosphorylation of TOR. Disruption of TOR signaling led to defects in BR-regulated growth, and there is substantial

overlap between TOR- and BR- regulated genes. Surprisingly, disruption of BR signaling through either chemical inhibition or genetic mutation induced autophagy, whereas enhanced BR signaling repressed starvation-induced autophagy. A fragment of TOR was shown to interact with BIN2 *in vitro*, and the adjacent fragment was phosphorylated by BIN2 kinase. Finally, multiple phosphorylation sites in the phosphorylated TOR fragment were identified by mass spectrometry. Our results suggest that BR regulates plant growth and autophagy through BIN2 phosphorylation of TOR, which reveals a new mechanism of interaction between the BR and TOR signaling pathways in the control of plant growth and stress responses.

3.2 Introduction

Brassinosteroids (BRs) are a major plant hormone family and are essential for regulation of plant growth and development (Li and Chory 1997). Deficiency in BR synthesis or signaling leads to growth and developmental defects including dwarfism and male sterility (Clouse, Langford et al. 1996, Li, Nagpal et al. 1996, Li, Nam et al. 2001). Application of exogenous BRs or gain-of-function mutants with enhanced BR signaling rescues the BR deficiency phenotype, resulting in plants with an increase in elongation of hypocotyls and petioles, vegetative growth, and seed production (Li and Chory 1999, Choe, Fujioka et al. 2001, Wang, Seto et al. 2001, Yin, Wang et al. 2002). The BR signaling pathway also interacts with multiple other signaling pathways to regulate plant growth and stress responses (Choudhary, Yu et al. 2012, Hao, Yin et al. 2013).

BRs are perceived by binding to Brassinosteroid-Insensitive 1 (BRI1), a leucine-rich repeat receptor-like kinase (LRR-RK) located in the plasma membrane (Li and Chory 1997,

Wang, Seto et al. 2001, Kinoshita, Cano-Delgado et al. 2005). In the absence of BRs, Brassinosteroid-Insensitive 2 (BIN2), a glycogen synthase kinase (GSK) -3-like kinase downstream of BRI1, phosphorylates two homologous transcription factors, BRI1-EMS-Supressor 1 (BES1) and Brassinazole-Resistant 1 (BZR1). This results in the inhibition of their function through cytoplasm retention, reduced DNA binding and degradation via the proteasome (He, Gendron et al. 2002, Li and Nam 2002, Vert and Chory 2006, Bai, Zhang et al. 2007, Gampala, Kim et al. 2007). In the presence of BR, BIN2 activity is inhibited (Li and Nam 2002, Yan, Zhao et al. 2009), and protein phosphatase 2A (PP2A) dephosphorylates and activates BES1 and BZR1 (He, Gendron et al. 2002, Wang, Nakano et al. 2002, Yin, Wang et al. 2002, Zhao, Peng et al. 2002, Tang, Yuan et al. 2011), Active BES1 and BZR1 accumulate in the nucleus and together regulate the expression of approximately 5000 genes (Yin, Vafeados et al. 2005, Sun, Fan et al. 2010, Yu, Li et al. 2011). PP2A also dephosphorylates BRI1 and inhibits BR signaling, suggesting that it participates in a negative feedback mechanism (Wu, Wang et al. 2011).

Recent studies suggested that phosphorylated BES1 and BZR1 can also be degraded through autophagy (Zhang, Zhu et al. 2016, Nolan, Brennan et al. 2017). Autophagy is a major pathway for degradation and recycling of cellular components and plays an important role in plant stress responses, senescence, and pathogen defense (Liu and Bassham 2012, Yang and Bassham 2015). Autophagy is maintained at a low basal level under non-stressed condition. In plant cells, autophagy is induced during senescence (Hanaoka, Noda et al. 2002), abiotic stresses such as nutrient starvation (Doelling, Walker et al. 2002), salt and drought stress (Liu, Xiong et al. 2009), oxidative stress (Xiong, Contento et al. 2007), ER stress (Liu, Burgos et al. 2012), and biotic stress such as pathogen infection (Liu, Schiff et al.

2005). Upon induction, a cup-shaped vesicle called a phagophore expands to engulf cargo to be degraded, and is completed into a double membrane autophagosome, which delivers the cargo into the vacuole for degradation and recycling (Yang and Bassham 2015). Many genes have been identified that are involved in autophagosome initiation and formation, and are named *autophagy-related* (*ATG*) genes (Tsukada and Ohsumi 1993, Yang and Bassham 2015). ATG8 is a core protein in the formation of autophagosomes, and is attached to the autophagosome membrane through conjugation to phosphatidylethanolamine (PE) (Ichimura, Kirisako et al. 2000). Increasing evidence indicates that autophagy can degrade specific organelles and proteins with the aid of adaptors, in a process termed selective autophagy (Svenning, Lamark et al. 2011, Zientara-Rytter, Lukomska et al. 2011, Zhou, Wang et al. 2013, Marshall, Li et al. 2015). We recently demonstrated that BES1 is targeted for selective autophagy through interaction of its adaptor DSK2 with ATG8, suggesting that selective autophagy functions in BR-regulated growth and stress responses (Nolan, Brennan et al. 2017).

Studies in yeast and mammals have identified many genes that function in the regulation of autophagy, although most of these do not exist in plants (Michaeli, Galili et al. 2016, Yin, Pascual et al. 2016). A key negative regulator of autophagy is target of rapamycin (TOR), a highly conserved Ser/Thr protein kinase that is present in yeast, animals and plants (Noda and Ohsumi 1998, Pattingre, Espert et al. 2008, Liu and Bassham 2010). The TOR signaling pathway is critical for coordination of cell division and growth, protein synthesis and metabolism, and autophagy induction for nutrient remobilization and stress tolerance (Dobrenel, Caldana et al. 2016). TOR signals through a complex composed of TOR, RAPTOR (regulatory-associated protein of TOR), which recruits substrates and presents

them to TOR for phosphorylation (Hara, Maruki et al. 2002), and LST8 (Lethal with Sec Thirteen 8), which stabilizes the TOR complex (Yang, Rudge et al. 2013). Disrupted TOR signaling leads to plant growth defects. An Arabidopsis knockout mutant of *TOR* is embryo lethal, while downregulation of *TOR* arrests plant growth and induces autophagy (Menand, Desnos et al. 2002, Liu and Bassham 2010). Knockout mutants in the TOR binding partners RAPTOR or LST8 also have severe growth defects and induced autophagy (Anderson, Veit et al. 2005, Deprost, Truong et al. 2005, Moreau, Azzopardi et al. 2012). Several TOR substrates have been identified in plants, including the E2Fa transcription factor, which regulates growth through regulation of transcription (Xiong, McCormack et al. 2013), ribosomal p70 S6 kinase (S6K), which regulates growth through translational regulation (Mahfouz, Kim et al. 2006, Xiong and Sheen 2012), and 2A phosphatase associated protein of 46 KD (TAP46), which interacts with PP2A and regulates autophagy (Ahn, Han et al. 2011).

A recent study suggested that TOR inhibits degradation of phosphorylated BZR1 through suppression of autophagy (Zhang, Zhu et al. 2016). BIN2 was also suggested to be regulated by TOR as a downstream effector for growth regulation (Xiong 2016). However, the mechanisms by which the BR and TOR signaling pathways interact to regulate plant growth and stress response remains unclear. In this study, we showed that disruption of TOR signaling caused defects in BR-regulated plant growth. Defective BR signaling led to autophagy induction, whereas enhanced BR signaling repressed autophagy during nutrient deficiency, suggesting that BRs act upstream of TOR to regulate autophagy. Surprisingly, and in contrast to previous research (Xiong, Zhang et al. 2016, Zhang, Zhu et al. 2016), our results indicated that TOR interacts with and is phosphorylated by BIN2, with multiple

phosphorylation sites detected by mass-spectrometry. These data place TOR downstream of BR signaling rather than upstream, and demonstrate a mechanism by which BR signaling modulates output of the TOR pathway. In summary, we identified a previously unknown interaction between the BR and TOR signaling pathways, providing a new mechanism of regulation of plant growth and stress responses.

3.3 Results

3.3.1 Disruption of the TOR signaling pathway compromises BR-regulated plant growth

We have recently shown that under stress conditions, the BR-regulated transcription factor BES1 interacts with ATG8 via the adaptor DSK2, leading to its degradation by autophagy (Nolan, Brennan et al. 2017). TOR kinase is a key regulator of plant growth and development, and also negatively regulates autophagy (Liu and Bassham 2010, Dobrenel, Caldana et al. 2016). Recent studies suggest that TOR can modulate signaling via the BR pathway (Xiong 2016, Zhang, Zhu et al. 2016); here we hypothesize that the converse may also occur, i.e. that BR signaling may modulate activity of the TOR signaling pathway. To test this hypothesis, we first assessed BR-regulated hypocotyl elongation in WT and a TOR signaling defective mutant, *raptor1b*. RAPTOR is a binding partner of TOR that recruits TOR substrates for phosphorylation (Hara, Maruki et al. 2002). Two isoforms of RAPTOR have been identified in Arabidopsis, RAPTOR1A and RAPTOR1B, with RAPTOR1B being more highly expressed in most organs (Anderson, Veit et al. 2005). Disruption of either TOR or RAPTOR1B leads to defects in plant growth and development, but knockout mutant of RAPTOR1A showed no difference compared to WT phenotype (Anderson, Veit et al. 2005,

Deprost, Yao et al. 2007, Montane and Menand 2013), suggesting RAPTOR1B plays a major role in plant TOR signaling. Seedlings of WT (Col-0), *raptor1b* and *bri1-301*, a previously characterized knock down mutant that has defects in BR signaling (Li and Nam 2002), were grown on medium with different concentrations of brassinolide (BL), the most active BR, for 12 days. Hypocotyl lengths were measured and normalized to the control with no BL (Figure 1A). The relative hypocotyl length increased in WT seedlings with increasing concentration of BL, suggesting that BR responses in WT increased with the BR level. The negative control *bri1-301* showed a significantly reduced BR response in low concentrations of BL, although the BR response increased to the same level as in WT at high concentrations of BL. In contrast, the *raptor1b* mutant had a significantly reduced BR response under all tested concentrations of BL, indicating that disrupting the TOR complex affects plant growth in response to BR.

To confirm that the reduced hypocotyl elongation in response to BR is due to a defect in TOR signaling, we assessed the effect of the TOR inhibitor AZD8055 (Dong, Xiong et al. 2015). WT seedlings were grown on medium with DMSO, 100 nM BL, 1 μM AZD8055, or both chemicals for 10 days, and hypocotyl lengths of seedlings were measured (Figure 1B). Compared to the DMSO treatment control, the addition of BL significantly increased hypocotyl length, whereas AZD8055 significantly reduced hypocotyl length. Consistent with the *raptor1b* mutant, the hypocotyl lengths with both BL and AZD8055 treatment were significantly reduced compared to BL alone, and showed no significant difference compared to the DMSO control. This suggests that defects in TOR signaling compromise BR-induced hypocotyl elongation, indicating that TOR is involved in BR-regulated plant growth.

BR signaling has been well characterized for its regulation of gene expression through transcription factors including BES1 and BZR1 (Guo, Li et al. 2013). TOR was also shown to function in regulation of gene expression in plants (Dong, Xiong et al. 2015). To test whether gene expression was affected by the interaction between TOR and BR signaling that leads to plant growth regulation, we compared the changes in the WT transcriptome after BL or AZD8055 treatment. Previous studies have collected whole transcriptome analysis data of BL or AZD8055 treatment through RNA-seq (Wang, Chen et al. 2014, Dong, Xiong et al. 2015). We analyzed the two RNA-seq data sets through clustering analysis of TOR induced or repressed genes with genes differentially expressed under BL treatment (Figures 1C and 1D). 29.3% of genes that are repressed by TOR (induced under AZD8055 treatment) also showed downregulation upon BL treatment, while 36% of genes induced by TOR (reduced under AZD8055 treatment) appeared to be upregulated by BL (Figure 1D). This suggests that the gene expression programs regulated by TOR and BR signaling pathways are related.

3.3.2 Defects in the BR signaling pathway induce autophagy

Although our results suggest that the TOR and BR signaling pathways are coordinated to regulate plant growth, the mechanism of interaction between the pathways remains unclear. TOR is also a negative regulator of autophagy in plants, and downregulation of TOR activates autophagy (Liu and Bassham 2010). A recent study suggested that the BR transcription factor BZR1 is degraded by autophagy, and that TOR might stabilize BZR1 by inhibition of autophagic degradation (Zhang, Zhu et al. 2016). Therefore, we hypothesized that TOR-regulated autophagy may be involved in BR signaling. To test this hypothesis, we

first examined the level of autophagy in BR signaling mutants (Figures 2A and 2B). *bin2-1D* is another mutant with BR signaling defects, and is a dominant gain-of-function mutant (Li, Nam et al. 2001). *bin2-T*, a triple knockout mutant of BIN2 and two BIN2 homologs, BIL1 and BIL2 (Yan, Zhao et al. 2009), and *bes1-D*, a dominant gain-of-function mutant with accumulation of BES1 (Vilarrasa-Blasi, González-García et al. 2014), both are mutants with enhanced BR signaling. WT, *bri1-301*, *bin2-1D*, *bin2-T* and *bes1-D* seedlings were grown under standard conditions for 7 days, transferred to sucrose-free medium and grown in darkness for an additional 3 days, or to sucrose-containing medium and grown in the light. Roots were then stained with the acidotropic dye monodansylcadaverine (MDC) (Biederbick, Kern et al. 1995, Contento, Xiong et al. 2005), and MDC-stained structures were observed and photographed by fluorescence microscopy. We have recently shown that in elongating Arabidopsis root cells, MDC staining extensively co-localizes with autophagosomes labeled with the specific marker GFP-ATG8e, validating the use of MDC as an autophagosome-selective marker under our conditions (Floyd, Morriss et al. 2015). MDC-stained autophagosomes appear as rapidly moving fluorescent puncta, and the number of autophagosomes in each image were counted for quantification (Figure 2B). As expected WT seedlings had a low level of autophagy under standard conditions, while autophagosome number significantly increased upon sucrose starvation. Surprisingly, both *bri1-301* and *bin2-1D* mutants had a significantly increased number of autophagosomes under both standard and sucrose-deficient conditions compared to WT under control conditions, although the induction of autophagy *bri1-301* was significantly lower than that in WT under sucrose starvation. The *bes1-D* gain-of-function mutant had no significant difference in the basal level of autophagy compared to WT under standard conditions, but had significantly

reduced autophagy induction upon sucrose starvation, suggesting that enhanced BR signaling represses autophagy induction under stress conditions. While another mutant with enhanced BR signaling, *bin2-T*, failed to repress autophagy induced by sucrose starvation, this might due to the presence of additional BIN2 isoforms functioning redundantly in the signaling pathway (Yan, Zhao et al. 2009). Our results indicated that defective BR signaling leads to constitutive autophagy, suggesting that BR signaling might be upstream of autophagy activation through regulation of TOR.

To confirm the MDC staining results, we used a transiently expressed mCherry-ATG8e fusion protein as a highly selective marker of autophagosomes. ATG8 is involved in the formation of autophagosomes, and fluorescent protein-tagged ATG8 has been broadly used as a marker of autophagosomes and autophagic bodies (Yoshimoto, Hanaoka et al. 2004, Contento, Xiong et al. 2005). WT, *bri1-301*, *bes1-D*, *bin2-1D*, *bin2-T* plants were grown in soil for 4-6 weeks under long-day conditions. Leaf protoplasts were isolated, transformed with mCherry-ATG8e, and incubated with or without sucrose for 2 days in darkness, followed by observation using fluorescence microscopy (Figure 2C). The percentage of protoplasts with active autophagy, defined as more than 3 visible autophagosomes or autophagic bodies in a protoplast (Yang, Srivastava et al. 2016), was calculated and averaged from 3 independent replicates. Consistent with the MDC staining results, both BR signaling defective mutants, *bri1-301* and *bin2-1D* showed constitutive autophagy under sucrose rich and deficient conditions, whereas *bes1-D* remained at an low basal level of autophagy, indistinguishable between sucrose-rich and -deficient conditions. The remaining BR signaling enhanced mutant, *bin2-T*, which behaved similarly to WT upon staining of roots with MDC (Figures 2A and 2B), showed repressed autophagy in leaf

protoplasts under sucrose starvation. The reason for this difference between root and leaf cells is unclear, but could potentially be due to different functions or expression of BIN2 homologs in different cell types (Xiong, Zhang et al. 2016). Taken together, disruption of BR signaling in the loss-of-function *bri1-301* mutant or the gain-of-function *bin2-1D* mutant leads to constitutive autophagy, whereas gain-of-function mutation of BES1 inhibits starvation-induced autophagy, suggesting that BR negatively regulates autophagy.

To further confirm that autophagy is regulated by BR signaling, we measured the extent of autophagy activation after addition of BL to increase BR signaling, or a BR biosynthesis inhibitor, brassinazole (BRZ), to inhibit BR signaling (Asami, Min et al. 2000). GFP-ATG8e-expressing seedlings were grown in standard conditions for 7 days and transferred to medium with or without sucrose, and plus DMSO, 100 nM BL or 1 μM BRZ for an additional 3 days. Seedlings growing on medium without sucrose were kept in the dark during treatment. Roots were observed by fluorescence microscopy, and the number of GFP-tagged autophagosomes in each image was counted for quantification (Figure 2D). Consistent with the results from BR signaling mutants, inhibition of BR signaling by BRZ significantly increased the level of autophagy under both control and sucrose starvation conditions compared to the basal level of autophagy in control conditions, and was not significantly different from sucrose starvation with DMSO. Meanwhile, enhancing BR signaling by application of BL caused a significant reduction in autophagy after sucrose starvation compared to addition of DMSO. This confirmed that disruption of BR signaling induces autophagy, while enhanced BR signaling represses starvation-induced autophagy.

3.3.3 Autophagy is regulated by BR signaling through the TOR signaling pathway

Our results showed that BR negatively regulates autophagy, suggesting that BR acts upstream of TOR in autophagy regulation, and hence BL should not repress autophagy upon inhibition of TOR. To test this, we examined sucrose starvation-induced autophagy in the presence of BL and the TOR inhibitor, AZD8055 (Figure 3A). 7-day-old GFP-ATG8e seedlings were transferred to medium with or without sucrose, plus DMSO, 100 nM BL, 1 μM AZD8055 or 100 nM BL plus 1 μM AZD8055 for an additional 3 days. Seedlings growing on medium without sucrose were kept in the dark during incubation. Roots of seedlings were observed by fluorescence microscopy, and the number of GFP-tagged autophagosomes in each image was counted for quantification. As expected, compared to autophagy induced by sucrose starvation in the presence of DMSO, the addition of BL significantly reduced starvation-induced autophagy. Inhibition of TOR by AZD8055 had significantly higher autophagy level under both sucrose-rich or -starvation conditions, compared to sucrose-rich condition with DMSO. Meanwhile, treatment with both BL and AZD8055 caused significantly induced autophagy under both sucrose rich and starvation conditions, and was indistinguishable from AZD8055 treatment alone. This showed that BL failed to inhibit autophagy induced by inhibition of TOR, supporting our hypothesis that BR represses autophagy through activation of TOR.

To test whether TOR is required for BR-regulated autophagy, we examined autophagy in WT and TOR signaling mutant leaf protoplasts with transiently expressed mCherry-ATG8e and YFP-BIN2-1D or YFP-BES1-D constructs. First we tested whether transient expression of BIN2-1D or BES1-D had the same effect on autophagy as stable expression in root cells of the mutants (Figure 3B). Leaf protoplasts of 4-week-old WT plants

were isolated and transformed with mCherry-ATG8e alone, or with YFP-BIN2-1D or YFP-BES1-D constructs. Transformed protoplasts were incubated with or without sucrose in darkness for 2 days, and the percentage of protoplasts with active autophagy was calculated. Expression of mCherry-ATG8e alone with sucrose does not induce autophagy, while co-expression of BIN2-1D with mCherry-ATG8e activated autophagy even under sucrose-rich conditions. In contrast, sucrose starvation induces autophagy in leaf protoplasts expressing mCherry-ATG8e alone, whereas co-expression of BES1-D and mCherry-ATG8 leads to reduced autophagy upon sucrose starvation.

Both *bin2-T* and *bes1-D* mutants showed repression of autophagy during sucrose deficiency (Figure 2C). BES1 is a BR transcription factor that is downstream of BIN2 in the BR signaling pathway (Guo, Li et al. 2013). To test whether *bin2-T* represses autophagy through BES1, and whether BES1 regulates autophagy through TOR, we examined whether expression of BES1-D can repress the constitutive autophagy observed in *bin2-1D* mutant protoplasts (Figure 3C). Leaf protoplasts from WT, *bin2-1D* and *raptor1b* mutant plants were isolated and transformed with mCherry-ATG8e with or without the YPF-BES1-D construct, followed by incubation with or without sucrose in the dark for 2 days. Autophagy was observed by fluorescence microscopy, and the percentage of protoplasts with active autophagy was calculated and averaged from 3 independent replicates. Autophagy was significantly induced in WT protoplasts after sucrose starvation compared to the control, and the overexpression of BES1-D significantly reduced autophagy in WT upon sucrose starvation. Both *bin2-1D* and *raptor1b* mutants showed constitutive autophagy under sucrose rich or deficient conditions compared to the basal level of autophagy in WT. Consistent with the results of AZD8055 treatment (Figure 1A), BES1-D failed to repress the constitutive

autophagy in the *raptor1b* mutant, suggesting that either TOR acts downstream of BES1 to regulate autophagy, or BES1 regulates autophagy through a TOR-independent mechanism. Interestingly, the expression of BES1-D also failed to repress the constitutive autophagy in *bin2-1D* mutant, suggesting that the activation of autophagy by BIN2-1D does not occur via BES1.

BIN2 is a GSK3-like kinase, and is an upstream regulator of BES1 in the BR signaling pathway (Youn and Kim 2015). Recent studies in mammalian cells suggest that GSK3 regulates TOR activity to control cell growth and development (Azoulay-Alfaguter, Elya et al. 2015, Stretton, Hoffmann et al. 2015). Therefore, we hypothesized that BIN2 negatively regulates TOR in Arabidopsis to control plant growth and autophagy. To test this hypothesis, YFP-BIN2-1D was transiently co-expressed with mCherry-ATG8e in WT, *bes1-D* and TOR-OE leaf protoplasts (Figure 3D). The TOR-OE line has been previously demonstrated to have increased *TOR* expression and enhanced growth (Ren, Qiu et al. 2011). Protoplasts were incubated without sucrose in darkness for 1 day and observed by fluorescence microscopy. The percentage of protoplasts with active autophagy was calculated and averaged from 3 independent replicates. Autophagy activity under control conditions in *bes1-D* and TOR-OE lines was indistinguishable from that in WT, while overexpression of BIN2-1D significantly induced autophagy in all genotypes. Consistent with the induced autophagy in overexpression of BES1-D in *bin2-1D* mutant, expression of BIN2-1D in the *bes1-D* mutant significantly increased autophagy, suggesting that BES1 cannot repress BIN2-induced autophagy. However, autophagy was also significantly induced by expression of BIN2-1D in the TOR-OE line, indicating that overexpression of TOR cannot repress BIN2-1D induced autophagy. This might due to posttranslational regulation of

BIN2 kinase inhibited the activity of overexpressed TOR, or that BIN2 regulates autophagy though both TOR and BES1. Taken together, our results suggest that BIN2 acts upstream of TOR to regulate autophagy, and that the BR transcription factor BES1 might have a redundant role with TOR signaling in BR-regulated autophagy.

3.3.4 BRs regulate the TOR signaling pathway through BIN2 phosphorylation of TOR

To explore how BIN2 regulates TOR, we first tested whether BIN2 directly regulates TOR through physical interaction. To produce recombinant TOR proteins in *E. coli*, we divided *TOR* into 4 fragments (Figure 4A), with each fragment containing about 500 amino acids. Each TOR fragment was cloned into the pET42a vector, with a GST tag at the N terminus. GST-tagged TOR fragments were incubated with recombinant BIN2-MBP (Yan, Zhao et al. 2009), GST or GST fusions captured with glutathione beads and analyzed by western blotting with MBP antibody (Figure 4B). These experiments showed that Fragment 2 of TOR could pull down BIN2-MBP. In contrast, no interaction was observed between BIN2-MBP and GST or BIN2-MBP and the other TOR fragments. These results indicate that BIN2 interacts with fragment 2 of TOR, which contains several HEAT repeats. HEAT repeats are a conserved domain in TOR among different species, and have been shown to mediate the interaction between TOR and RAPTOR in Arabidopsis (Mahfouz, Kim et al. 2006). This suggests that this domain functions in protein-protein interaction, and is likely to be the region interacting with BIN2.

Given the interaction between TOR and BIN2, we next tested whether TOR is phosphorylated by BIN2 kinase. An *in vitro* phosphorylation assay using recombinant GST and GST-tagged TOR fragments demonstrated that fragment 3 of TOR (GST-TOR-F3) could

be strongly phosphorylated by BIN2 kinase (Figure 4C). This suggested that BIN2 phosphorylates TOR, and that the phosphorylation sites are within the region of fragment 3. Taken together, this showed that TOR interacts with BIN2 through HEAT repeats in fragment 2 and is phosphorylated by BIN2 kinase within fragment 3.

Finally, to identify the BIN2 phosphorylation sites within TOR, TOR fragment 3 was phosphorylated by GST-BIN2 *in vitro* and analyzed by mass spectrometry (Table 1). Several phosphorylated peptides were identified, whereas no TOR phosphorylation was detected in a control lacking BIN2. Four of the detected phosphorylated residues, T1154, T1155, T1157, and S1161, were in a region containing BIN2 consensus phosphorylation sites T1157-Q-Q-L-S1161 (S/T-X-X-X-S/T) (Zhao, Peng et al. 2002), (Figure 4A), suggesting that BIN2 phosphorylates TOR at and around these amino acids. Taken together, our results suggest that BIN2 interacts with and phosphorylates TOR to regulate plant growth and autophagy (Figure 4D).

3.4 Discussion

Plants have evolved complicated signaling networks to coordinate growth and stress responses for surviving under constantly changing environmental conditions, and BRs are one of the plant hormones that stimulate plant growth and interact with multiple signaling pathways for defense of stresses (Chaiwanon, Wang et al. 2016). TOR is also playing a central role in balancing plant growth and stress responses (Henriques, Bogre et al. 2014). In this study, we showed a new interaction between the BR and TOR signaling pathway through BIN2 phosphorylation of TOR. Defects in the TOR signaling by chemical treatment or genetic modification lead to compromised hypocotyl elongation, suggesting TOR plays an important role for BR-regulated plant growth. Previous studies showed that inhibition of

TOR lead to differential gene expression regulated by plant hormones, including BRs (Dong, Xiong et al. 2015). Our clustering analysis with previous transcriptome between BR and TOR regulated genes displayed a high percentage of overlap, suggesting the two pathways have a strong correlation in regulation of plant growth by regulation of the transcriptome.

Autophagy plays an important role in stress response via recycling of cellular materials, and is negatively regulated by TOR (Liu and Bassham 2012). BR transcription factors, BES1/BZR1, inhibit BR signaling upon phosphorylation and degradation through ubiquitination (Li and Jin 2007). Recent studies suggest BZR1 can be degraded through autophagy (Zhang, Zhu et al. 2016), and BES1 is degraded by selective autophagy through interaction with ATG8 (Nolan, Brennan et al. 2017), suggesting autophagy plays a role in regulation of hormonal signaling. Interestingly, our results showed that defects in BR signaling lead to autophagy induction, whereas enhanced BR signaling repress starvation induced autophagy, suggesting BR is also an upstream regulator of autophagy in response to stresses, and BR signaling might regulate autophagy through a negative feedback mechanism.

We characterized that BRs regulate autophagy through interaction between BIN2 and TOR, that is BIN2 is an upstream negative regulator of TOR. Gain-of-function mutant *bes1-D* repressed autophagy induced by nutrient deficiency, but failed to repress autophagy upon TOR inhibition. In addition, *bin2-1D* showed constitutive autophagy that cannot be repressed by BES1-D transgene or overexpression of TOR, suggesting BES1 and TOR might function redundantly in BR-regulated autophagy, although *bin2-1D* induced autophagy remained induce in TOR overexpression plants might due to a stronger effect of posttranslational regulation of active BIN2 kinase. However, further investigation is required to identify the

mechanism of BES1 regulation of autophagy. Furthermore, BIN2 has been suggested to be involved in BR singling regulation of stress response (Youn and Kim 2015), although the mechanism remained unclear. Our results therefore provide a potential mechanism for BIN2 regulation of stress response through regulation of autophagy.

Our results provided evidences showing BIN2 directly interact with and phosphorylates TOR *in vitro*, with multiple phosphorylation sites of TOR identified by mass-spectrometry. The peptides identified most phosphorylation residues are in the region around BIN2 consensus phosphorylation sites (S/T-X-X-X-S/T) (Zhao, Peng et al. 2002), supporting that the identified residues are due to phosphorylation by BIN2. However, how BIN2 phosphorylate TOR to inhibit TOR signaling remains unclear. BIN2 is a GSK3-like kinase that regulate downstream through phosphorylation (Li and Nam 2002). Previous studies in yeast and mammals showed GSK3 inhibit TOR through phosphorylation of the tuberous sclerosis complex (TSC) (Inoki, Ouyang et al. 2006, Rallis, Townsend et al. 2017). However, homolog of TSC appears to be missing in plants. Another TOR upstream regulator in animals for nutrient and energy sensing is AMP activated protein kinase (AMPK) (Dobrenel, Caldana et al. 2016), which also inhibits TOR through phosphorylation of TSC (Inoki, Zhu et al. 2003). Interestingly, studies in human showed that AMPK can also phosphorylate RAPTOR to inhibit TOR complex function in TSC-deficient cell types (Gwinn, Shackelford et al. 2008). The plant homolog of AMPK, Snf1-related protein kinase 1 (SnRK1), also showed signaling functions in plants (Hulsmans, Rodriguez et al. 2016). However, the AMPK phosphorylated residues on mammalian RAPTOR is not conserved in plants, suggesting plant TOR might develop a mechanism for inhibition by SnRK1. AMPK and GSK3 have been shown act in parallel pathways for inhibition of TOR in yeast (Rallis, Townsend et al.

2017), suggesting a similar function mechanism between them. Therefore, plant TOR might also have developed a mechanism for BIN2 phosphorylation and inhibition. However, future work is needed to confirm the phosphorylated residues on TOR by the BIN2 kinase, and to characterize how BIN2 inhibit TOR activity.

Studies in animal and human have also shown that GSK3 could act as a positive upstream regulator of TOR through phosphorylation of TSC or RAPTOR (Zhou, Freeman et al. 2013, Azoulay-Alfaguter, Elya et al. 2015, Stretton, Hoffmann et al. 2015), and TOR also negatively regulates GSK3 through S6K (Parisi, Riccardo et al. 2011, Wang, Brown et al. 2011), which might due to distinct function of GSK3 in different cell types or conditions. Recent studies in plants also showed that BIN2 acts downstream of TOR through S6K phosphorylation (Xiong 2016), suggesting a negative feedback mechanism of BIN2-TOR signaling. Taken together, our study revealed that TOR is phosphorylated by BIN2 for regulation of plant growth and autophagy, and therefore providing a new interaction between BR signaling and the TOR signaling pathway for coordination of plant growth and stress response.

3.5 Materials and methods

Plant materials and growth conditions

Arabidopsis seeds of WT (Col-0) or indicated genotypes were sterilized with 33% [v/v] bleach and 0.1% [v/v] Triton X-100 [Sigma] for 20 min, followed by 5 washes of 5 minutes each time with sterile water. Sterilized seeds were stored at 4°C in darkness for at least 2 days to allow stratification before plating on solid ½ MS medium (2.22g/L Murashige-Skoog vitamin and salt mixture [Caisson Laboratory, MSP09], 1% [w/v] sucrose,

0.6% [w/v] Phytoblend agar [Caisson Laboratory], 2.4 mM 2-morphinolino-ethanesulfonic acid [MES], pH 5.7). Seedlings were grown under long-day conditions (16 h light) at 22 °C for 7 days. Plants for transient expression in leaf protoplasts were grown in soil in a humidity-controlled growth chamber with 50% humidity at 20-23 °C under long-day conditions for 4 to 6 weeks. For sucrose starvation, 7-day-old seedlings grown on solid ½ MS medium were transferred to solid ½ MS medium lacking sucrose and kept in darkness for an additional 3 days.

Mutants and transgenic lines used in this study include: *raptor1b* (salk_078159), GFP-ATG8e (Xiong, Contento et al. 2007), TOR-OE (Ren, Qiu et al. 2011), *bri1-301* (Li and Nam 2002), *bin2-1D* (Li, Nam et al. 2001), *bin2-3 bil1 bil2* (*bin2-T*) (Yan, Zhao et al. 2009), and *bes1-D* (Vilarrasa-Blasi, González-García et al. 2014).

Characterization of BR response phenotype

Sterilized Arabidopsis seeds were germinated and grown on solid ½ MS medium with DMSO, brassinolide (BL) or AZD8055 at the indicated concentrations in the light for 10 days. Plates were photographed and hypocotyl length was measured using ImageJ (Schneider, Rasband et al. 2012).

Gene expression analysis

Previously reported RNA-seq data for 4-week old WT plants treated with or without BL were used to analyze the effect of BL on TOR-regulated genes (Wang, Chen et al. 2014, Dong, Xiong et al. 2015). TOR- and BL-regulated gene lists were compared using Venny (http://bioinfogp.cnb.csic.es/tools/venny/index.html). Significance of observed gene overlaps

was assessed using the Genesect tool in VirtualPlant (Katari, Nowicki et al. 2010). Reported overlaps were statistically significant at a $P< 0.05$ level. Clustering was performed using the 'aheatmap' function of the NMF package in R (https://cran.r-project.org/web/packages/NMF/index.html) using log2 reads per million mapped reads (RPM) values.

Autophagy detection by fluorescence microscopy

Arabidopsis seedling roots were stained with monodansylcadaverine (MDC, Sigma) as described previously (Contento et al., 2005). MDC-stained seedlings were observed and imaged using a Zeiss Axio Imager.A2 upright microscope (Zeiss) equipped with a Zeiss Axiocam BW/color digital cameras using a DAPI-specific filter at the Iowa State University Microscopy and Nanoimaging Facility. GFP-ATG8e transgenic seedlings were observed and photographed with the same fluorescence microscopy system using a GFP-specific filter. Cells within the root elongation zone were photographed and the number of autophagosomes in each image was counted and averaged from at least 10 images per sample. Confocal microscopy images of autophagosomes in root cells or leaf protoplasts were taken using a Leica SP5 × MP confocal/multiphoton microscope system (Leica) with a 63x/1.4 oil immersion objective at the Iowa State University Roy J. Carver High Resolution Microscopy Facility (Pu and Bassham 2016).

Transient expression in protoplasts

mCherry-ATG8e was transiently expressed alone in Arabidopsis leaf protoplasts as previously described (Sheen 2002), or with YFP-BIN2-1D or YFP-BES1-D constructs. 25-30 μg of each plasmid DNA was introduced into protoplasts using 40% (w/v) polyethylene

glycol (PEG, Sigma-Aldrich). Protoplasts were washed and incubated in W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES, pH 5.7) without sucrose for starvation stress, or with 0.5% (w/v) sucrose as control at room temperature in darkness for 2 days in total. Protoplasts were observed by fluorescence microscopy (Nikon Eclipse E200) using a FITC filter, and protoplasts with more than 3 visible autophagosomes were counted as active for autophagy (Yang, Srivastava et al. 2016). A total of 100 protoplasts were observed per genotype for each condition, and the percentage of protoplasts with induced autophagy was calculated and averaged from 3 independent experimental replicates.

Generation of constructs

The *TOR* coding region was divided into 4 fragments for amplification, and each TOR fragment was amplified from Col-0 cDNA with primers including restriction sites as listed in Supplemental Table 1. Fragments 1 and 3 were digested with EcoRI and SalI, and fragments 2 and 4 were digested with NcoI and SalI. Digested fragments were fused with a GST tag at the N-terminus in the pET42a(+) vector (Novagen).

Protein-protein interaction assays

Recombinant proteins were produced in *E. coli* strain BL21, and GST-pull down experiments were carried out as described previously (Yin, Wang et al. 2002). GST or each GST-tagged TOR fragment was incubated with BIN2-MBP (Yan, Zhao et al. 2009) in 1mL GST-pulldown buffer (50mM Tris-HCl pH 7.5, 200mM NaCl, 0.5% Trition X-100 and 0.5 mM β-mercaptoethanol, Roche complete mini protease inhibitor cocktail) at room temperature for 2 hours on a tube rotator. Following incubation, 20 μL GST beads pre-

blocked overnight with 1mg/mL BSA and BL21 extract were added and the incubation was continued for an additional 30 minutes. GST beads were washed in GST-pulldown buffer 5-6 times and then eluted in 2X SDS sample buffer. BIN2-MBP was detected with anti-MBP antibodies (NEB) (Yin, Wang et al. 2002).

TOR *in vitro* phosphorylation assays

Each GST-tagged TOR fragment, or GST alone as control, were incubated with GST-BIN2 kinase in 20 μL of kinase buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 12 mM MgCl2 and 10μCi ^{32}P-γATP) and *in vitro* kinase assays carried out as described previously (Yin, Wang et al. 2002). For mass spectrometry analysis of phosphorylated proteins, GST tagged TOR fragment 3 (GST-TOR-F3) was phosphorylated using GST-BIN2 in kinase buffer containing 10 mM unlabeled ATP. Proteins were run on Phostag-SDS PAGE gels and shifted bands excised after silver staining or digested directly in solution as described below (Kinoshita, Kinoshita-Kikuta et al. 2006).

Protein digestion and LC-MS/MS

Proteins were reduced with 5 mM TCEP in ammonium biocarbonate for 5 min at 94 °C. Proteins were then digested using either Glu-C (ThermoFisher) or trypsin (Roche) at 37 °C overnight and then alkylated with 12.5 mM iodacetamide for 15 min at 37 °C in the dark. Peptides were further digested using an additional aliquot of Glu-C or trypsin for 2 hrs. Samples were then acidified to a pH of ~3 with formic acid. Digested peptides were purified using Waters Oasis MCX cartridges and eluted using 45%IPA/500mM NH_4HCO_3. Eluted

peptides were dried using a speedvac (Thermo) and resuspended in 0.1% formic acid. Peptide amount was then quantified using the Pierce BCA Protein assay kit.

An Agilent 1260 quaternary HPLC was used to deliver a flow rate of ~600 nL min-1 to a 3-phase capillary chromatography column through a splitter. The 3-phase capillary chromatography was assembled as follows. Using a Next Advance pressure cell a fused silica capillary column was packed with 5 μM Zorbax SB-C18 (Agilent) to form the first dimension reverse phase column (RP1). A 5 cm long strong cation exchange (SCX) column packed with 5 μm PolySulfoethyl (PolyLC) was connected to RP1 using a zero dead volume 1 μm filter (Upchurch, M548) attached to the exit of the RP1 column. A nanospray fused silica capillary was pulled to a sharp tip using a laser puller (Sutter P-2000) and packed with 2.5 μM C18 (Waters) to form RP2 and then connected to the SCX column. The 3 sections were joined and mounted on a custom electrospray source for on-line nested elutions. A new set of columns was used for every sample. Peptides were loaded onto RP1 using the Next Advance pressure cell. Peptides were eluted from RP1 unto the SCX column using a 0 to 80% acetonitrile gradient over 60 min. Peptides were then fractionated using the SCX column using a series of 9 salt gradients (0, 30, 50, 60, 70, 80, 90, 100 and 1000 mM ammonium acetate), followed by high resolution reverse phase separation using an acetonitrile gradient of 0-80% for 150 min.

Spectra were acquired on a Thermo Scientific Q-Exactive high-resolution quadrupole Orbitrap mass spectrometer. Data dependent acquisition was obtained using Xcalibur 3.0.63 software in positive ion mode with a spray voltage of 2.00 kV and a capillary temperature of 275 °C. MS1 spectra were measured at a resolution of 70,000, an automatic gain control (AGC) of 3e6 with a maximum ion time of 100 ms and a mass range of 400-2000 m/z. Up to

15 MS2 were triggered at a resolution of 17,500, an AGC of 1e5 with a maximum ion time of 50 ms and a normalized collision energy of 28. MS1 that triggered MS2 scans were dynamically excluded for 15 s.

The raw data were extracted and searched using Spectrum Mill v4.01 (Agilent Technologies). MS/MS spectra with a sequence tag length of 1 or less were considered to be poor spectra and were discarded. The remaining MS/MS spectra were searched against the Arabidopsis TAIR10 proteome. The enzyme parameter was limited to tryptic peptides with a maximum mis-cleavage of 2. Carbamidomethylation was set as a fixed modification while Ox-Met, and phosphorylation on Serine, Threonine, or Tyrosine were defined as variable modifications. A maximum of 6 phosphorylation events per peptide was used. A 1:1 concatenated forward-reverse database was constructed to calculate the false discovery rate (FDR). The tryptic peptides in the reverse database were compared to the forward database, and were shuffled if they matched to any tryptic peptides from the forward database. Cutoff scores were dynamically assigned to each dataset to obtain a peptide false discovery rate (FDR) of 0.1%. Phosphorylation sites were localized to a particular amino acid within a phosphopeptide using the variable modification localization (VML) score in Agilent's Spectrum Mill software (Chalkley and Clauser 2012).

Accession numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative under the following accession numbers: TOR, AT1G50030; RAPTOR1B, AT3G08850; ATG8e, AT2G45170; BRI1, AT4G39400; BIN2, AT4G18710; BES1, AT1G19350.

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3.8 Figures and tables

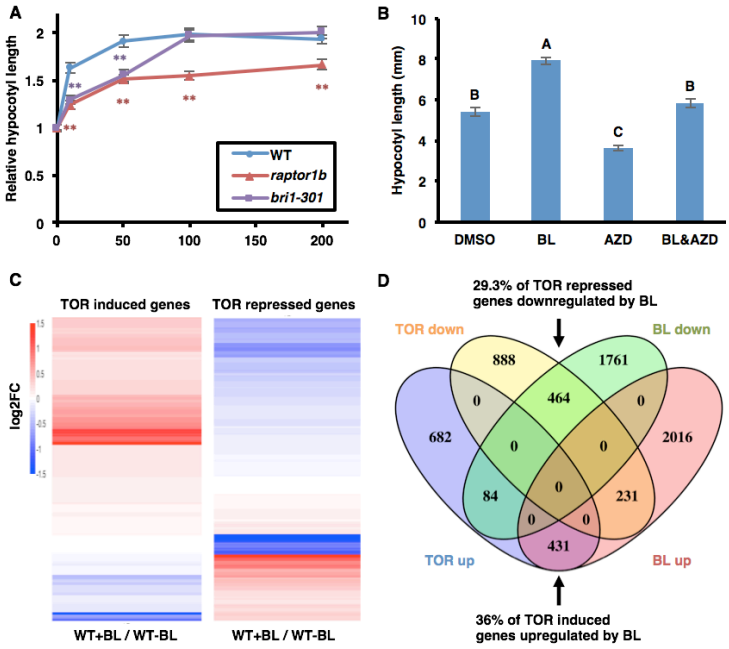

Figure 1. BR-regulated plant growth is arrested upon disruption of TOR signaling.

(A) Response of WT (Col-0), *raptor1b* and *bri1-301* seedlings to brassinolide (BL). Seedlings of each genotype were grown on ½ MS medium with the indicated BL concentration for 12 days. Hypocotyl lengths were measured and averaged from 27-35 seedlings and normalized to the control of no BL treatment. Shown is a representative graph of one of three independent replicates. Data represent means ± standard error (SE) and asterisks indicate statistically significant differences (**$P < 0.01$) using Student's *t*-test compared with WT under the same conditions. **(B)** Hypocotyl elongation in response to BL or AZD8055 (AZD). WT seedlings were grown on ½ MS medium with 100 nM BL, 1 μM AZD8055, or both chemicals for 10 days. Hypocotyl lengths of 18-30 seedlings were measured and averaged from 3 independent replicates. Data represent means ± standard error (SE). Different letters indicate statistically significant differences ($P < 0.05$) using Student's *t*-test. **(C)** Clustering analysis of TOR-regulated genes in response to BL treatment from whole transcriptome RNA-seq data. Previously reported RNA-seq data for 4-week old WT plants treated with or without BL were analyzed with VirtualPlant. Clustering analysis was performed with the NMF package in R. Color depicts log2 fold change of BL treatment compared to the control. **(D)** Comparison of TOR and BL-regulated genes in whole transcriptome RNA-seq. Gene lists from **(C)** were compared using Venny.

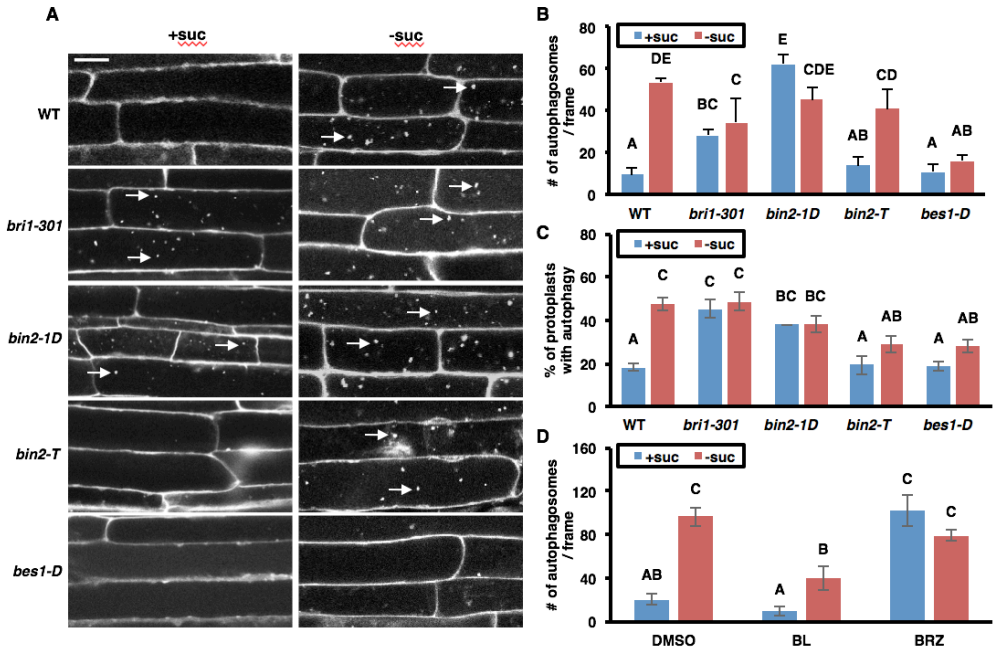

Figure 2. Defects in BR signaling lead to autophagy induction.

(A) Representative confocal images of MDC-stained autophagosomes. 7-day-old WT, *bri1-301*, *bin2-1D*, bin2-T and BES1-D mutant seedlings were transferred to solid ½ MS medium with sucrose in the light, or without sucrose in the dark for an additional 3 days. Treated seedlings were stained with MDC. MDC-stained autophagosomes appear as white puncta within cells as indicated by the arrows. Scale bar = 25 μm **(B)** Quantification of the number of MDC-stained autophagosomes in each genotype treated as in A. Seedlings were stained with MDC and images were captured using fluorescence microscopy. The number of autophagosomes in each image was counted and averaged from 10 images per genotype for each condition. **(C)** Transient expression of mCherry-ATG8e in leaf protoplasts of the indicated genotype for each condition. Leaf protoplasts were incubated with or without sucrose for 2 days, and observed using fluorescence microscopy. The percentage of protoplasts with more than 3 visible mCherry-tagged autophagosomes was calculated, with 100 protoplasts observed per genotype for each condition. **(D)** Quantification of the number of GFP-tagged autophagosomes under each condition. 7-day-old GFP-ATG8e seedlings were transferred to solid ½ MS medium plus 100 nM brassinolide (BL), 1 μM brassinazole (BRZ) or DMSO as control for an additional 3 days. Seedlings were observed using fluorescence microscopy, and quantified as in **(B)**. All graphs represent means ± standard error (SE). Different letters indicate statistically significant differences ($P < 0.05$) using Student's *t*-test.

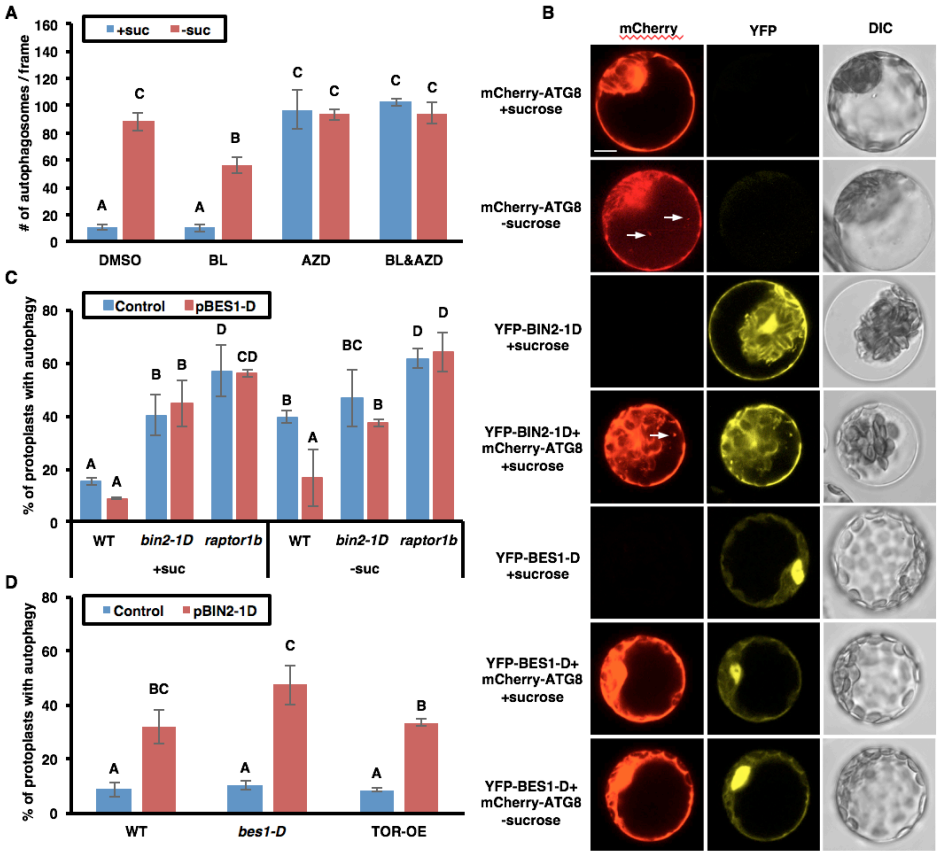

Figure 3. BR regulates autophagy through TOR-dependent and TOR-independent pathways

(A) Quantification of the number of GFP-tagged autophagosomes under the indicated conditions. 7-day-old GFP-ATG8e seedlings were transferred to solid ½ MS medium with or without sucrose, plus 100 nM brassinolide (BL), 1 μM AZD8055 (AZD), both BL and AZD, or DMSO as control for an additional 3 days. Seedlings were observed using fluorescence microscopy and images captured. The number of autophagosomes in each image was counted and averaged from 10 images for each condition. **(B)** Representative confocal images of co-expression of mCherry-ATG8e and YFP-BIN2-1D or YFP-BES1-D. Leaf protoplasts were incubated with or without sucrose for 2 days, and observed using confocal microscopy. Autophagosomes are indicated by the arrows. Scale bar = 10 μm. **(C)** Quantification of autophagy in protoplasts with co-expressed YFP-BES1-D (pBES1-D) and mCherry-ATG8e in the indicated genotype for each condition. **(D)** Quantification of autophagy in protoplasts of the indicated genotype with co-expressed YFP-BIN2-1D (pBIN2-1D) and mCherry-ATG8e. For all graphs, data represent means ± standard error (SE). Asterisks or different letters indicate statistically significant differences ($P < 0.05$) using Student's *t*-test.

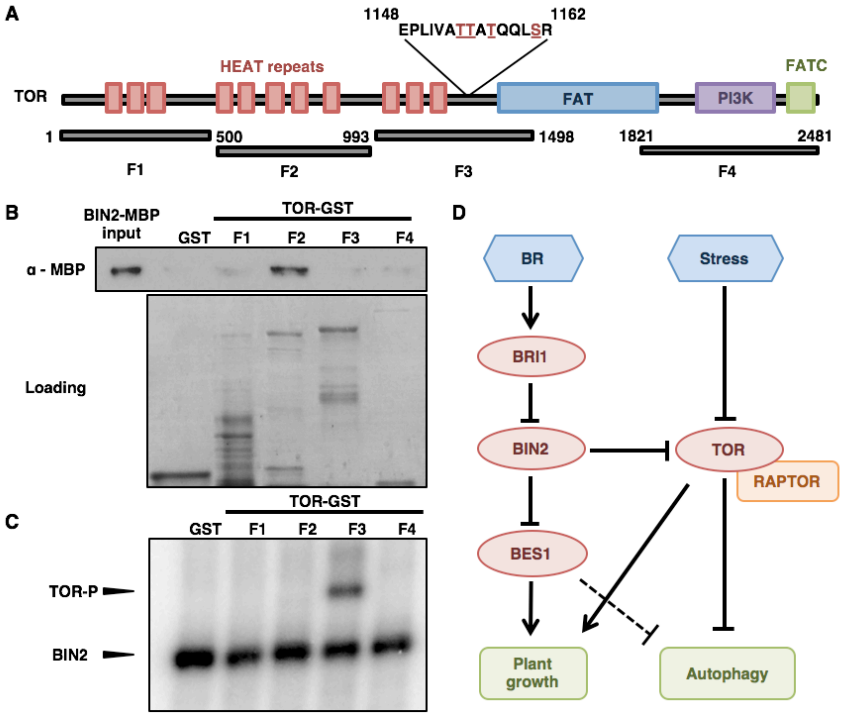

Figure 4. TOR is phosphorylated by BIN2 kinase.

(A) Schematic diagram of conserved domains, cloned fragments and identified phosphorylation sites of *TOR*. F1-F4, fragments 1-4. Numbers indicate amino acid positions. Putative BIN2 phosphorylation sites identified by mass-spectrometry are underlined and colored in red **(B)** GST-pull down of GST-tagged *TOR* fragments after incubation with BIN2-MBP. BIN2-MBP was detected with anti-MBP antibodies. Loading indicates amounts of GST proteins used in the pulldown assay. **(C)** Phosphorylation of TOR fragments by BIN2 in an *in vitro* phosphorylation assay. Arrows indicate the phosphorylated TOR fragment or BIN2 autophosphorylation. **(D)** Schematic model of interactions between the BR and TOR signaling pathways. In the absence of BRs, BIN2 kinase phosphorylates and inactivates both BES1 and TOR to inhibit growth and activate autophagy. When BRs are present, they are perceived by the BRI1 receptor, leading to inhibition of BIN2 kinase and promotion of growth. BRs likely regulate autophagy through multiple mechanisms including BIN2 phosphorylation of TOR and regulation of autophagy components by BES1 or downstream transcription factors (dashed arrow).

Table 1. Identified phosphorylation sites in TOR fragment 3 by mass-spectrometry following *in vitro* phosphorylation by BIN2.

TOR Phosphorylation Site(s)	# of Phosphorylated Residues	Peptide Sequence
T996 or Y997	1	(996)TYLPVILPCFIQVLGDAER(1014)
T1065	1	(1061)DAIKTLTR(1068)
T1154 or T1555 or T1157 or S1161	1	(1147)REPLIVATTATQQLSR(1162)
T1154 or T1555 or T1157 or S1161	4	(1148)EPLIVAttAtQQLsRRLPVEV IRDPVIENEIDPFEEGTDR(1187)
T1185	1	(1163)RLPVEVIRDPVI ENEIDPFEEGtDR(1187)
S1401 or T1403 or T1404 or T1413	1	(1400)ASQTtNPHLVLEATLGQMR(1418)
S1438	1	(1436)YWsPAEPSARLE(1447)
S1470	1	(1446)LEMAPMAAQAAWNMGE WDQMAEYVsRLDDGDETK(1479)

Recombinant proteins of GST-TOR-F3 was phosphorylated by GST-BIN2 through *in vitro* phosphorylation assay. Phosphorylation sites were identified through mass-spectrometry. When phosphorylation sites could not be localized due to peptide fragmentation patterns, all possible residues are listed. Number of phosphorylated residues indicates sites detected within a single peptide. No phosphorylation was detected in mock phosphorylation reactions lacking BIN2.

Supplemental Table 1. Primers used for generating the constructs containing fragments of *TOR*. Restriction sites are underlined.

Gene	Forward	Reverse
TOR F1	CACCGAATTCATGTCTACCTCGTCG CAATCTTTTGT	CACCGTCGACGGCTAGTTGAACTTG AGCTGAACAA
TOR F2	CACCCCATGGAGCTGCAGACTCTTG CTCGTTTCAATT	CACCGTCGACTTCATCATTAAGTGC CAAGCAAAGA
TOR F3	CACCGAATTCTTCAGAACTTATCTT CCAGTCATCCTTCCA	CACCGTCGACGAATGTGCCATTACT GCTCCCATCG
TOR F4	CACCCCATGGAGACAGCTGATGTCC AAACCGCATTGAAGAC	CACCGTCGACTCACCAGAAAGGGCA CCACCCAAC

CHAPTER 4

IRE1B LINKS ER STRESS TO AUTOPHAGY IN ARABIDOPSIS BY DEGRADING RNAS ENCODING PROTEINS THAT NEGATIVELY REGULATE AUTOPHAGY

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4.1 Abstract

Autophagy is a conserved process in eukaryotes that contributes to cell survival in response to stress. Previously, we found that ER stress induces autophagy via a pathway dependent upon IRE1b, an ER membrane-associated factor involved in the splicing of *bZIP60* mRNA. IRE1 is a dual protein kinase and ribonuclease, and here we determined the

involvement of the protein kinase catalytic domain, nucleotide binding and RNase domains of IRE1b in activating autophagy. Autophagy was assessed in transgenic seedlings bearing mutations in the various IRE1b domains, and nucleotide binding and RNase activity of IRE1b are required for ER stress-mediated autophagy. The RNase activity is involved in IRE1b mRNA splicing function, but its principal splicing target, *bZIP60*, is not involved in IRE1b induced autophagy, and we therefore considered other roles for IRE1b in the activation of autophagy. Clustering of ER localized IRE1b-YFP was observed when seedlings were subjected to ER stress, but the RNase knockout mutation in IRE1b still undergoes clustering, suggesting that IRE1b clustering does not induce autophagy. Upon ER stress, the RNase of IRE1 has been found to engage in another activity called Regulated Ire1-Dependent Decay of Messenger RNA (RIDD), which is the promiscuous degradation of mRNA by IRE1 in response to ER stress. 12 RIDD target genes were selected from RNA-seq data for testing their role in inhibiting autophagy, and three of them, β-glucosidase 21 (β-GLU21), MD2 related lipid recognition protein (ML) and peroxidase 14 (PR14) are suggested to be negative regulators of autophagy induced by ER stress.

4.2 Introduction

Environmental stresses are a major threat to optimal productivity of plants in agricultural settings (Sah, Reddy et al. 2016), and multiple simultaneous stresses are often encountered. Plants activate a suite of protective and adaptive mechanisms to respond to these stresses, including transcriptional, metabolic and cellular adaptations that often limit growth while increasing stress tolerance and survival (Dong and Chen 2013). One such mechanism is the upregulation of a degradation pathway termed autophagy, in which cellular

materials are transferred inside the vacuole, degraded by the resident vacuolar lytic enzymes, and the breakdown products recycled (El-Din El-Assal, Le et al. 2004, Yang and Bassham 2015). Autophagy is active at a basal level under normal growth conditions, but is highly upregulated by a wide variety of biotic and abiotic stresses (Blom, Gammeltoft et al. 1999, Ichimura, Kirisako et al. 2000, Xiong, Contento et al. 2005, Xiong, Contento et al. 2007, Liu, Xiong et al. 2009, Liu, Burgos et al. 2012, Kwon, Cho et al. 2013, Zhou, Wang et al. 2013, Dobzinski, Chuartzman et al. 2015). During nutrient deficiency it functions in recycling of materials for re-use of the breakdown products to enable cell survival (Moriyasu and Ohsumi 1996, Hara, Maruki et al. 2002, Sarbassov, Ali et al. 2004, Xiong, Contento et al. 2005, Díaz-Troya, Florencio et al. 2008), whereas in other conditions it appears to degrade oxidized and aggregated proteins and other macromolecules to prevent toxicity (Ichimura, Kirisako et al. 2000, Xiong, Contento et al. 2007, Thomas 2013, Zhou, Wang et al. 2013).

In plants, autophagy is initiated by the recruitment of the autophagy-related protein ATG5 to the endoplasmic reticulum (ER), which results in membrane expansion to form a bowl-shaped double-membrane intermediate, the phagophore (Xie, Liu et al. 2016). Membrane expansion continues until sealing of the phagophore to generate a double-membrane vesicle termed an autophagosome (Liu, Schiff et al. 2005, Díaz-Troya, Florencio et al. 2008), along with release from the ER. The source of membrane for autophagosome formation is still unclear, but may derive from newly-synthesized lipids originating at the ER as well as membrane trafficked from pre-existing organelles including the ER, Golgi, endosomes and plasma membrane (Cheng, Willmann et al. 2002, Farmer, Rinaldi et al. 2013, Escamez, Andre et al. 2016, Zhang, Zhu et al. 2016). Cargo for degradation is incorporated into the forming autophagosomes either non-selectively or via binding by a receptor protein

that recruits cargo by interacting with the autophagosome membrane protein ATG8 (Gagne, Downes et al. 2002, Floyd, Morriss et al. 2012, Kelley and Estelle 2012, Zhou, Wang et al. 2013, Marshall, Li et al. 2015). The autophagosome outer membrane then fuses with the tonoplast, releasing the inner membrane and contents into the vacuole for degradation and recycling (Yang and Bassham 2015).

In Arabidopsis, autophagy is upregulated by ER stress, a condition in which unfolded or misfolded proteins accumulate in the lumen of the ER. ER stress can be induced by agents that prevent proper folding of ER proteins, including DTT and tunicamycin (TM) (Howell 2013, Liu and Howell 2016). Under ER stress conditions, but not other autophagy-inducing conditions such as starvation, fragments of ER are delivered to the vacuole by autophagy (Liu, Burgos et al. 2012), potentially as a mechanism for disposal of aggregates of unfolded proteins that cannot be degraded by other proteolysis pathways (Liu and Bassham 2013). ER stress-induced autophagy is activated by the accumulation of misfolded proteins in the ER, as the effect can be moderated by either chemical or molecular chaperones and can be triggered by expression of chronically misfolded proteins (Yang, Srivastava et al. 2015).

ER stress elicits the Unfolded Protein Response (UPR), in which plants activate signaling pathways that result in the upregulation of genes that function in protein folding, the degradation of misfolded ER proteins, and the degradation of ER-associated mRNAs to reduce the protein load at the ER (Liu and Howell 2016). There are two arms of the UPR signaling pathway in Arabidopsis. One arm involves bZIP28, a membrane-bound transcription factor that is retained in the ER under normal conditions by its interaction with the molecular chaperone BiP (Srivastava, Deng et al. 2013). In response to ER stress, bZIP28 is transported to the Golgi, where it is processed and released from the membrane, allowing it

to move into the nucleus and activate transcription of ER stress response genes (Liu, Srivastava et al. 2007, Gao, Brandizzi et al. 2008, Tajima, Iwata et al. 2008). The second arm involves the unconventional splicing factor IRE1, which splices the mRNA encoding the transcription factor bZIP60. Unspliced bZIP60 mRNA encodes a membrane-localized protein, whereas splicing by IRE1 leads to loss of the transmembrane domain and the acquisition of a nuclear targeting signal. The spliced form can therefore enter the nucleus and activate transcriptional responses (Deng, Humbert et al. 2011, Nagashima, Mishiba et al. 2011, Chen and Brandizzi 2012). IRE1 also functions in the degradation of mRNAs encoding secretory proteins (Mishiba, Nagashima et al. 2013). Arabidopsis contains two active IRE1 isoforms, IRE1a and IRE1b, which overlap substantially in function but also have some functional specificity (Koizumi, Martinez et al. 2001, Liu, Burgos et al. 2012, Moreno, Mukhtar et al. 2012). We have shown previously that IRE1b is required for activation of autophagy during ER stress, whereas disruption of IRE1a has little effect on autophagy (Liu, Burgos et al. 2012).

IRE1 is a dual-function ribonuclease and protein kinase (Howell 2013). Point mutations in Arabidopsis IRE1b disrupting either its ribonuclease or kinase activities indicated that, as expected, IRE1'sribonuclease is required for bZIP60 splicing. Autophagy can be induced in response to ER stress in an Arabidopsis *bzip60* mutant (Liu, Burgos et al. 2012), suggesting that IRE1b's role in linking ER stress to autophagy is independent of bZIP60 splicing. In animal cells, activation of autophagy upon ER stress requires the kinase activity of IRE1, which activates the c-Jun N-terminal kinase pathway, in turn upregulating autophagy (Ogata, Hino et al. 2006). However, homologs of Jun kinase and components of its signaling pathway are absent in plants.

Here, we demonstrate that the catalytic activity of IRE1b's protein kinase is dispensable for ER stress-induced autophagy, but its ribonuclease activity is required, although independent of its normal downstream splicing target, bZIP60 mRNA. Instead, we identify mRNAs that inhibit the activation of autophagy, indicating that their degradation by IRE1b's Regulated IRE1-dependent RNA degradation (RIDD) activity (Hollien, Lin et al. 2009) is required for the upregulation of autophagy during ER stress.

4.3 Results

4.3.1 bZIP60 is not required for the IRE1b-dependent induction of autophagy by ER stress

We have previously shown that IRE1b is required for linking ER stress to autophagy (Liu, Burgos et al. 2012); we therefore considered several possible mechanisms by which IRE1b might serve in this role (Figure 1A). One possibility is that IRE1b splices bZIP60 mRNA to make an active form of bZIP60 that upregulates genes required for the induction of autophagy. A second is that, through its protein kinase activity, IRE1b might autophosphorylate itself or phosphorylate other factors, initiating a signaling cascade leading to the induction of autophagy. Third, upon activation, IRE1b may oligomerize and cluster in the ER membrane, and the clustering might promote autophagy. Fourth, in response to ER stress, IRE1b might attack other mRNAs through its RIDD activity, (Hollien, Lin et al. 2009), with the destruction of these RNAs being required for the induction of autophagy.

Since bZIP60 is a strong transcription factor (Iwata and Koizumi 2005) and its mRNA is the principal target of IRE1's mRNA splicing activity, we reexamined the issue as to whether bZIP60 functions downstream from IRE1b in linking ER stress to autophagy.

Earlier studies by Liu et al (Liu, Burgos et al. 2012) addressed this matter using a loss-of function mutant, *bzip60-1*. In the interim, however, it was reported that *bzip60-1* is a weak allele with respect to certain phenotypes (Zhang, Chen et al. 2015). *bzip60-1* is a T-DNA mutant with an insertion in the first exon of the gene. However, an in-frame ATG downstream from the T-DNA insertion may be functional (Figure 1B), and we observed a modest level of partial transcript accumulation in *bzip60-1* representing the second exon, but not the full first exon (Figure 1C). Another allele, *bzip60-3*, is an intron insertion, and we observed low level accumulation of partial transcripts representing the first exon, but not the second. The third allele, *bzip60-2*, has an insertion in the second exon and like *bzip60-3* we found modest accumulation of partial transcripts bearing the first exon. Although all three mutants accumulated partial transcripts, *bzip60-2* is considered to be a strong allele because the T-DNA insertion disrupts the IRE1 splice site, preventing the formation of a functional transcription factor targeted to the nucleus (Zhang, Chen et al. 2015).

When autophagy was examined in the roots of the *bzip60* mutants treated with TM, ER stress induced levels of autophagy in all the mutants comparable to WT as assessed by the formation of autophagosomes (Figure 1D and 1E). Thus, we concluded that in accordance with earlier findings, bZIP60 is not required for the IRE1b-dependent induction of autophagy by ER stress.

4.3.2 Kinase activity of IRE1b is not involved in autophagy induction upon ER stress

The second mechanism we explored was whether IRE1b might phosphorylate other proteins initiating a signaling cascade culminating in the induction of autophagy. To test this possibility, we used site-specific mutations in IRE1b to disrupt various IRE1 functions. The

site-specific mutations in the cytoplasmic domain of IRE1b were developed and described by Deng et al (Deng, Srivastava et al. 2013) (Figure 2A). The double D608N K610N mutation disrupts the nucleotide-binding site in IRE1b. It has been demonstrated in yeast that nucleotide binding is a critical step in activating IRE1's RNase activity (Papa, Zhang et al. 2003). D628A is in the protein kinase catalytic domain and blocks the phosphor-transfer activity of IRE1b (Deng, Srivastava et al. 2013). N820A is in the RNase domain and disables both the RNA splicing and RIDD functions of IRE1b. The RNase activity of IRE1b mutants in protoplasts have been tested by RT-PCR of spliced bZIP60 mRNA (Figure 2D). RNase activity was disrupted in the *ire1a ire1b* double knock out mutant, nucleotide binding domain mutant, and RNase domain mutants, while remained active in WT and other mutants.

IRE1 has protein kinase activity and in other organisms undergoes auto-transphosphorylation upon activation of IRE1 (Shamu and Walter 1996, Lee, Dey et al. 2008, Ali, Bagratuni et al. 2011). In metazoans, IRE1 activates Apoptosis Signal–regulating Kinase 1(ASK1), initiating a phosphorylation cascade leading to the activation of Jun amino-terminal kinase (JNK) (Ichijo, Nishida et al. 1997). However, IRE1 in yeast is not known to phosphorylate any other cellular proteins. In Arabidopsis, the D628A mutation in the catalytic site of IRE1 kinase domain knocks out its phospho-transfer activity (Nishitoh, Saitoh et al. 1998, Matsukawa, Matsuzawa et al. 2004, Tabas and Ron 2011, Deng, Srivastava et al. 2013, Shiizaki, Naguro et al. 2013). This mutation was tested for its support of ER stress-induced autophagy by transient expression in Arabidopsis leaf protoplasts derived from *ire1a ire1b* mutant leaves. As controls, protoplasts from both WT and *ire1a ire1b* mutants were mock-treated or treated with the ER stress agent TM. In mock-treated WT protoplasts only about 15% of cells showed evidence of ER stress-induced autophagy

(cells with >3 autophagosomes per cell) (Figure 2B and 2C). On the other hand, more than 50% of WT protoplasts displayed significant levels of autophagy when treated with 5 μg/ml TM. In *ire1a ire1b* mutant protoplasts, mock treatment or TM treatment resulted in the same background level of autophagy, little more than 15% of the cells showing >3 autophagosomes per cell.

When protoplasts from *ire1a ire1b* were transfected with non-mutant IRE1b constructs driven by the CaMV 35S promoter, complementation of the double mutant to nearly full WT levels of autophagy was observed. Similar results were obtained when *ire1a ire1b* protoplasts were transfected with D628A IRE1 constructs, lacking protein kinase activity. Nearly full complementation by constructs encoding IRE1b with disabled protein kinase activity indicated that the phospho-transfer activity of IRE1b is not required for ER stress to induce autophagy. Similar results were obtained with stably-transformed seedlings (Figure 3). Therefore, phosphorylation of other proteins by Arabidopsis IRE1b, if it does occur, is not involved in the induction of autophagy by ER stress.

4.3.3 The ribonuclease activity of IRE1b is required for autophagy induction upon ER stress

The RNase activity of plant IRE1 mediates the splicing of bZIP60 mRNA (Deng, Srivastava et al. 2013). The RNase domain of IRE1 is located near its C-terminus, and the N820A mutation in the catalytic site inactivates its endonuclease activity (Deng, Srivastava et al. 2013). As above, a N820A IRE1b construct was similarly transfected into *ire1a ire1b* protoplasts and tested for its ability to restore ER stress-induced autophagy. Unlike D628A, the RNase-dead construct failed to complement the double mutant *ire1a ire1b* mutant and

restore ER stress induced autophagy in transient assays with protoplasts (Figure 2B and 2C) and in stably transformed seedlings (Figure 3). This suggested that RNase activity of IRE1b is necessary for activation of autophagy upon ER stress.

It has been shown in other systems that the RNase activity of IRE1 is dependent on nucleotide binding, but not necessarily on the phospho-transfer activity of the protein kinase. For example, Papa et al. (Papa, Zhang et al. 2003) showed that a form of IRE1 with an expanded nucleotide-binding pocket is activated by an ATP mimic and kinase inhibitor, 1NM-PP1. Other studies demonstrated that the RNase activity of wild-type IRE1 can also be activated *in vitro* by other kinase inhibitors, such as APY29 and Sunitinib (Korennykh et al., 2009). The binding of ATP-mimetic ligands to the nucleotide-binding site of IRE1 promotes its oligomerization, which, in turn, activates its RNase activity. Thus, it is the ligand occupancy in the nucleotide-binding site that drives the conformational changes activating IRE1 (Mendez, Alfaro et al. 2015). In our system, we determined whether a functional nucleotide-binding site in IRE1b was required for ER stress induction of autophagy. We used the double D608N K610N mutant to disrupt the nucleotide-binding site in IRE1b (Deng, Srivastava et al. 2013). In the transient expression system (Fig. 2B and 2C) and in stably transformed seedlings (Figure 3), D608N K610N mutants failed to restore ER stress-induced autophagy to *ire1a ire1b*. Thus, we reason that nucleotide binding to IRE1b is needed to activate IRE1's RNase activity in order to link ER stress to autophagy.

4.3.4 IRE1 clustering is not sufficient for autophagy induction upon ER stress

Nucleotide binding activates IRE1 by altering its conformation and promoting its oligomerization or clustering (Shamu and Walter 1996, Kimata, Ishiwata-Kimata et al.

2007). Oligomerization is vital to IRE1's function because it promotes transphosphorylation of IRE1 monomers and creates surfaces for the binding of the target mRNA (Korennykh, Egea et al. 2009). IRE1 undergoes clustering in yeast and mammalian cells in response to stress (Li, Korennykh et al. 2010), and we wanted to know whether IRE1b clusters in Arabidopsis and whether clustering promotes autophagy. To test this, we developed an IRE1b-YFP fusion construct and demonstrated that it is biologically active shown by its support for the upregulation of BIP3 expression in response to stress (Figure 4C). When transfected into protoplasts, IRE1b-YFP was distributed in protoplasts in a pattern similar to an ER marker, as expected (Figure 4A). When the protoplasts were treated with TM, we observed pronounced clustering of IRE1b-YFP. Similar YFP fusions were generated with the RNase dead IRE1b to make IRE1b (N820A)-YFP (Figure 4B). This construct was not biologically active, as expected, as demonstrated by its failure to upregulate BIP3 in response to stress (Figure 4C). When the RNase dead fusion construct was introduced into protoplasts, it clustered normally in response to treatment by stress. Because the RNase dead fusion construct clustered normally, but did not support ER stress induced autophagy, this strongly indicated that IRE1b clustering is not sufficient for ER stress-induced autophagy.

4.3.5 RIDD genes negatively regulate induction of autophagy upon ER stress

Having eliminated the first three possibilities for the mechanisms by which IRE1b mediates the link between ER stress and autophagy, we explored the possibility that the RIDD activity of IRE1b might be involved in this role. RIDD targets in Arabidopsis have been identified by Mishiba et al. (Mishiba, Nagashima et al. 2013) and Deng et al. (Deng, Srivastava et al. 2013) as genes with expression that declines in response to ER stress in WT,

but not in *ire1a ire1b* mutants. We performed a transcriptomic analysis and identified the top 12 genes that were most highly downregulated in *ire1a ire1b* compared to WT and also in *ire1b* alone compared to WT (Figure 5A and Supplemental Table S1). The most vulnerable targets are mRNA transcripts encoding secretory proteins, associated with ribosomes during synthesis on the ER membrane.

The involvement of RIDD in mediating ER-stress induced autophagy could be interpreted to mean that certain RNA transcripts have to be degraded by IRE1b to allow ER stress to induce autophagy. Therefore, we overexpressed each of the top RIDD targets to determine whether they would interfere with ER stress induced autophagy in a WT background. cDNAs representing the RIDD target mRNAs were introduced into Arabidopsis protoplasts and autophagosome production in response to treatment with tunicamycin was assessed. We found that the expression of cDNAs representing β-glucosidase 21 (β-GLU21), peroxidase 14 (PR14) and ML (MD2 related lipid recognition protein) were most disruptive to ER stress-induced autophagy in this transient expression system (Figure 5B).

It is possible that either the RNA or the protein that it encodes might interfere with the induction of autophagy. Therefore, we generated mutant forms of β-GLU21, PR14 and ML in which the initiating AUG was knocked out, and tested them for their ability to disrupt ER stress-induced autophagy (Figure 5C). We found that none of the mutants interfered with ER stress-induced autophagy, indicating that the protein product encoded by the RNA, rather than the RNA itself was most likely responsible for the interference. To further confirm the results in the transient expression system, we also examined autophagy in stably-transformed seedlings (Figure 5D). Three transgenic lines of each gene were examined for autophagy induction by fluorescence microscopy, and showed significantly reduction of autophagy

upon ER stress compared to WT or the empty vector control 2301S. Taken together, our results suggest β-GLU21, PR14 and ML are negative regulators of ER stress-induced autophagy.

4.4 Discussion

Autophagy is generally regarded as a cell survival or renewal response that functions by turning over cellular contents. Autophagy in response to ER stress is thought to degrade damaged ER components since ER stress induces the formation of autophagosomes that include ER membranes and their contents. It was shown previously in Arabidopsis that IRE1b is required for activation of autophagy specifically during ER stress (Liu, Burgos et al. 2012). We considered possible mechanisms by which IRE1b might function in autophagy, and distinguished between them by knocking out bZIP60 and by selectively disabling various IRE1b activities. We eliminated several possibilities, including the involvement of bZIP60, a protein phosphorylation cascade initiated by the protein kinase activity of IRE1b, and the stress induced clustering of IRE1b. Loss of bZIP60 had no effect on autophagy, nor did a D628A mutation in IRE1b which inhibits its phospho-transfer activity. By contrast, a N820A mutation in the RNase domain of IRE1b effectively blocked ER stress-induced autophagy, demonstrating the essential role of the RNase activity. In addition, disruption of the nucleotide binding site in IRE1b by the D608N K610N mutation had the same effect. The likely explanation for this is that nucleotide binding, but not phospho-transfer is required to activate the RNase activity of IRE1.

Because IRE1b RNase activity, but not bZIP60, is required to link ER stress to autophagy, it suggests that the RIDD activity and not the RNA splicing activity of IRE1 is

important. In view of these findings, we assessed whether the RNA degradation that links ER stress to autophagy is global or selective with respect to the transcripts degraded. To address this issue, we tested the top 12 RIDD targets individually for their ability to disrupt ER stress-induced autophagy. The transcripts from three genes negatively regulated ER stress-induced autophagy, β-GLU21, ML and PR14, whereas the others had no effect. Knocking out the major open reading frames in these transcripts demonstrated that the proteins encoded by the transcripts, and not the RNAs themselves, were critical for inhibiting autophagy.

The three genes identified that enable ER stress-induced autophagy have different, and not well described, ER functions. At1g66270 (β-GLU21) is a member of the β-glucosidase family, a major component of ER bodies (Hayashi, Yamada et al. 2001). ER bodies are large spindle-shaped structures that are contiguous with the ER and are produced constitutively in seedlings, but are wound-induced in rosette leaves (Matsushima, Hayashi et al. 2002), where they are thought to function in defense responses (Nakano et al 2016). Upon encountering stresses such as salt stress, ER bodies fuse with the vacuole, delivering stress-response and cell death components into the vacuole (Hayashi et al., 2001). β-GLU21 itself is not wound inducible, but is a component of ER body contents. β-glucosidase family members have been implicated in ER body formation; the *nai1* Arabidopsis mutant downregulates β-GLU23 and lacks constitutive ER bodies, while another mutant that downregulates β-GLU18 prevents the formation of wound inducible ER bodies (Ogasawara, Yamada et al. 2009). β-GLU21 is closely related to β-GLU23, and whereas single loss-of-function β-GLU21 mutants have no ER body phenotype, they synergize with β-GLU23 mutants in reducing ER bodies (Nagano, Maekawa et al. 2009), suggesting that these proteins work together in ER body formation. It is not known whether glucosidases are recruited into

ER bodies as they form or whether they assemble ER bodies. In either case, the β-glucosidases have a role in the assembly of large membrane-bound structures such as ER bodies. Autophagosome formation also requires large quantities of membrane components. Under stress conditions, whether ER body glucosidases such as β-GLU21compete with assembly of other vesicular structures from the ER, such as autophagosomes, needs further investigation.

The other two genes that negatively act upon ER stress induced autophagy encode proteins that interact with lipids. At2g16005 is a MD2-related lipid recognition domain protein (ML), and proteins with this domain often bind and/or transport lipids or sterols (Inohara and Nunez 2002). It is also known as *ROSY1* (*InteractoR Of SYnaptotagmin1*), a gravity-regulated gene that binds stigmasterol and phosphoethanolamines (Dalal, Lewis et al. 2016). The third gene identified, At5g01870, is a pathogenesis related protein (PR14) related to lipid transfer proteins, and is unrelated in sequence to ML proteins. Lipid transfer proteins provide non-vesicular means by which lipids can be transferred from the ER to other organelles. Autophagosome formation involves the lipidation of ATG8 achieved by its coupling to phosphatidylethanolamine (Ichimura, Kirisako et al. 2000), and also requires the recruitment of lipids to the newly-forming autophagosome membrane. It is possible that these lipid-interacting proteins negatively regulate autophagy by competing or interfering with the lipidation of autophagic factors or by disrupting the recruitment or assembly of lipid components in the formation of autophagosomes.

An important question is whether the transcripts of these genes are the incidental or intended targets of IRE1b. In our work and that of Mishiba et al. (2013) in Arabidopsis, the major RIDD targets are RNA transcripts encoding proteins that enter the endomembrane

system. These transcripts are loaded onto ribosomes and serve as templates for the synthesis of proteins undergoing co-translational insertion into the ER. During stress, the degradation of RNAs encoding secreted proteins by IRE1 may reduce the load of proteins in the ER requiring folding. The slowing of translation during ER stress is well documented in metazoans in which PERK phosphorylates and inactivates eIF2a, which in turn reduces translation initiation (Harding, Zhang et al. 1999). Arabidopsis has no identifiable PERK homolog, and instead, RIDD may serve to slow translation during ER stress.

If the role of IRE1b in regulating ER stress-induced autophagy is to eliminate factors that interfere with the induction of autophagy, then what actually induces autophagy in response to stress? Is it simply the elimination of these negative regulators that leads to the induction of autophagy? Or does IRE1b function as a "licensing factor" that renders cells competent to respond to positive induction signals? The problem is similar to the induction of autophagy in response to metabolic deprivation. Future work is needed to characterize the mechanism of how IRE1b regulates autophagy upon ER stress.

4.5 Materials and Methods

Plant materials and growth conditions

All lines used in this study were in the Columbia-0 (Col-0) background. Seeds were sterilized in 33% (v/v) bleach with 0.1% (v/v) Triton X-100 for 20 min, followed by washing with sterile water at least five times. After being stratified in the dark at 4°C for at least 48h, sterilized seeds were plated and germinated on ½ strength MS solid media (Murashige & Skoog vitamin and salt mixture [Caisson, http://www.caissonlabs.com], 0.5 % [w/v] Sucrose, 2.4 mM MES, pH 5.7, and 0.6% [w/v] phytoagar). Unless otherwise noted, plants were

grown at 22°C in long-day conditions (16h-light/8h-dark). *bip60-1*, *bzip60-2*, *ire1b* and*ire1a ire1b*mutants and transgenic lines harboring various IRE1b point mutants were described previously (Deng et al. 2013). The *bzip60-3* (GABI-Kat, 326A12) mutant was obtained from Nottingham Arabidopsis Stock Centre and GABI-Kat (Kleinboelting et al., 2012), and homozygous plants were genotyped using gene specific primers and the T-DNA specific primer pAC161-LB1. Primers used in this study are listed in Supplemental Table S2.

MDC staining and microscopy analyses

MDC staining of TM treated Arabidopsis root was described in detail by (Contento et al., 2005; Liu et al., 2012). Seven-day-old seedlings were treated with DMSO (as the control) or 5μg/mL TM in 1/2 MS liquid medium for 6h, followed by incubation with 0.05 mM MDC for 10 min in the dark. After three brief washes with PBS, samples were observed using a Carl Zeiss microscope, and the 4', 6-diamidino-2-phenylindole-specific filter was used to visualize MDC fluorescence. For confocal microscopy analysis, samples were observed with a Leica SP5 X MP confocal microscope. A 4',6-diamidino-2-phenylindole-specific filter was used for visualizing the fluorescence of MDC, and a fluorescein isothiocyanate-specific filter for visualizing the GFP fluorescence.

Vector construction

For cloning the 12 RIDD target genes, PCR fragments were first amplified with gene-specific primers (Supplemental Table S2) from Col-0 cDNA by using PfuUltra II Fusion HS DNA Polymerase (Agilent, Cat. No. 600670-51). Then PCR fragments of β-GLU21, VSP1, PROX34, PROX-P, PR14, CTS1, GLH19, MD2, PROX-S were digested with Bam H I and

Sal I; and fragments of PR4 and PME41 were digested with Bam H I and Xho I. After being cleaned using a QIAquick Gel Extraction Kit (QIAGEN, Cat. No. 28704), clean digested fragments were inserted into pCambia2300 vector (Hajdukiewicz et al., 1994) by DNA Ligation Kit Mighty Mix (Takara, Cat. No. 6023), which contains a double 35S promoter to drive expression and a downstream NOS terminator.

For constructs used for analysis of IRE1bclustering, PCR fragments of IRE1b and IRE1bN820A were amplified directly from the constructs in our former study (Deng et al., 2013), digested with Sma I and Spe I, and inserted into a modified pCambia1300S-YFP vector with a double 35S promoter and NOS terminator.

Subcellular localization analyses

Protoplast isolation and transformation were previously described (Sheen, 2002). For analysis of IRE1b-YFP and IRE1bN820A-YFP clustering upon ER stress, 20μg plasmids (GenElute HP Plasmid Maxiprep Kit, Cat. No. NA0310-1KT, Sigma, USA) at a concentration of 1μg/μL were introduced into WT protoplasts. After dark incubation for 12h, protoplasts were treated with DMSO (as control) or with 5μg/mL TM for 6h in 6-well COSTAR Cell Culture Plates (Corning Incorporated, USA). For co-localization analyses, 20μg of IRE1b-YFP with 20μg ER-mCherry (TAIR stock number CD3-959) or Golgi-mCherry (TAIR stock number CD3-967) (Nelson et al., 2007) were co-transformed into WT protoplasts and incubated in the dark for 12h before observation by confocal microscopy. For visualization of autophagosomes, GFP-ATG8e was introduced alone or together with various IRE1b constructs. Images were taken using a Leica confocal microscope using a ×63 Leica

oil immersion objective, with excitation and emission at 520 nm and 550 nm for YFP, 488 nm and 509 nm for GFP, and 575 nm and 650 nm for mCherry.

RNA isolation and RT-PCR analyses

RNA samples were extracted using an RNeasy Plant Mini Kit (QIAGEN, Cat. No. 74904), according to the manufacturer's instructions. Synthesis of the first strand cDNA was performed with the iScript™ cDNA Synthesis Kit (BioRad, USA). RT-PCR was performed on C1000 Touch Thermal Cycler (BioRad, USA) in 8-well strip PCR tubes. Primers used in RT-PCR are listed in Supplemental Table S2.

RNA-seq analysis

Seeds from WT, *ire1b* and *ire1a ire1b*genotypes were germinated and seedlings grown vertically on 100 mm × 100 mm square plates (Fisher Scientific) on 1/2 MS medium for 7 days. Seedlings of similar size were treated with DMSO (as the control) or with 5µg/mL TM in 6-well cell culture plates with liquid 1/2 MS-0 media for 6h. Samples were harvested by removal of the liquid and immediately ground into powder in liquid nitrogen. Total RNA was extracted using an RNeasy Plant Mini Kit (QIAGEN, Cat. No. 74904). Quality and quantity of RNA were assessed by using a Nano drop1000 (Thermal Scientific, USA), and the ratio of absorbance for samples with both OD 260/230 and OD 260/280 > 1.8 were sent for RNA sequencing by BGI-Hongkong.

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3.8 Figures and tables

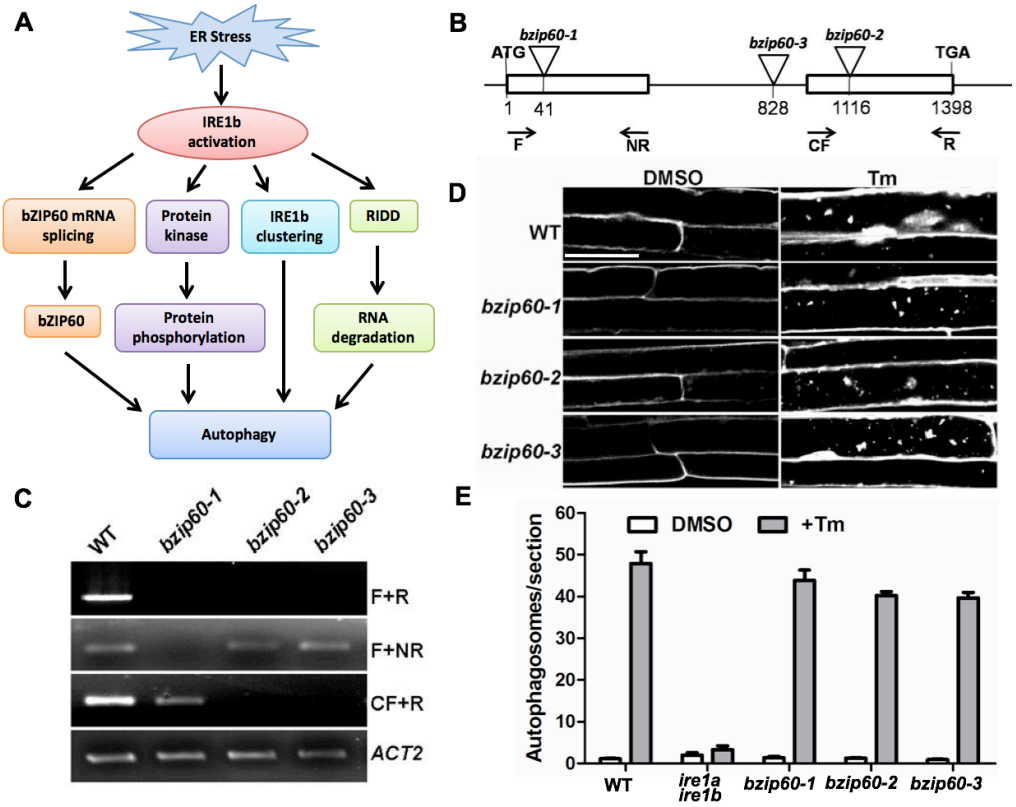

Figure 1. ER Stress induced autophagy in Arabidopsis seedling roots is independent of *bZIP60*.

(A) Possible mechanisms by which IRE1b links ER stress to autophagy, either by bZIP60 splicing, protein phosphorylation, IRE1b clustering or Regulated IRE1-dependent DNA Degradation (RIDD). **(B)** Schematic diagram of T-DNA insertion in *bzip60* mutants. Exons and intron are depicted to scale by boxes and line, respectively. The positions of T-DNA insertions are indicated by triangles, the numbers below showed the insertion sites or start and stop codons in base pair units, and the arrows below indicated primer binding sites. **(C)** RT-PCR analysis of *bZIP60* gene expression in wild-type (WT) and the three *bzip60* mutants using the primers depicted in **(B)**. Total RNA was isolated from 7-day-old seedlings. *ACTIN2* (ACT2) was used as a loading control. **(D)** Seven-day-old WT and three *bzip60* mutants were transferred to 1/2 MS liquid medium plus DMSO as control, or supplemented with 5 µg/mL TM for 6 hours to induce ER stress. Autophagosomes were visualized by MDC staining and confocal microscopy. Bar = 50 µm. **(E)** The number of autophagosomes per root section in A was assessed following TM treatment and staining by MDC. Error bars represent SE, n > 20.

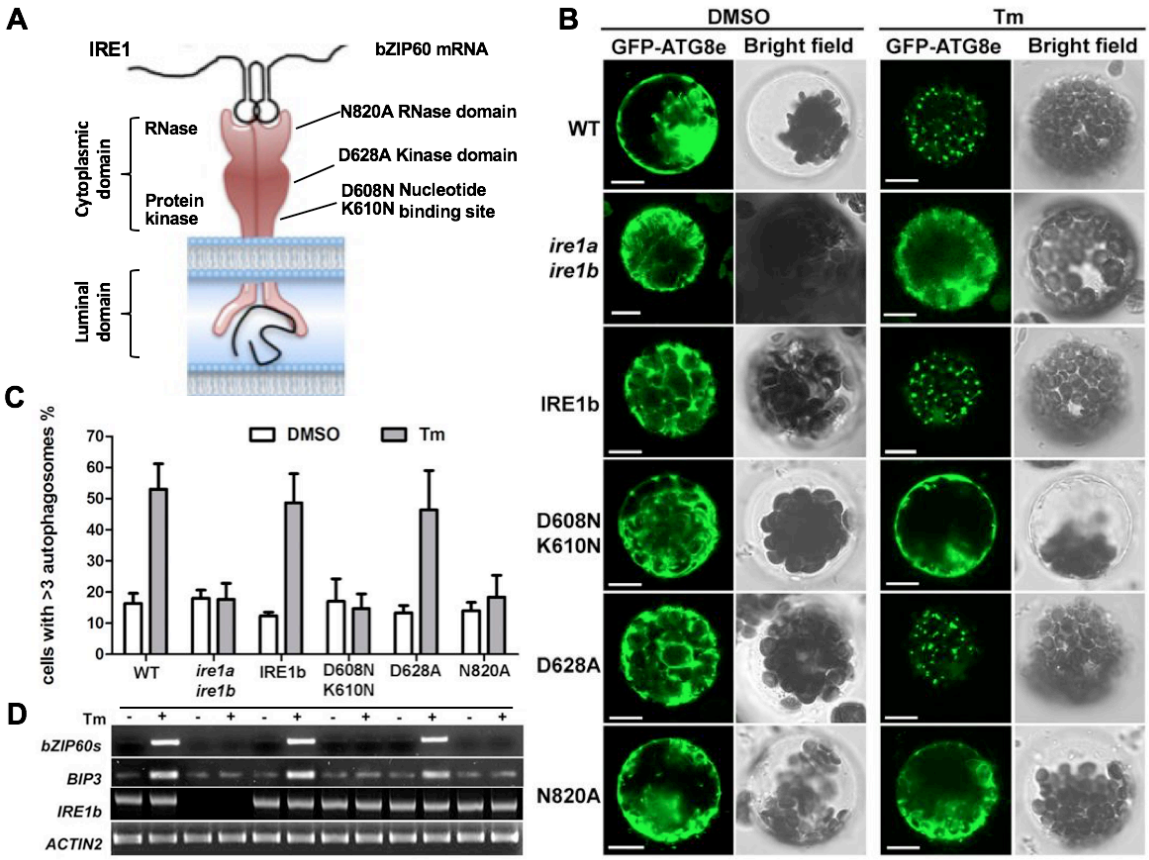

Figure 2. Complementation of autophagy defect in *ire1a ire1b* protoplasts by various *IRE1b* constructs.

(A) illustration of the disposition of IRE1b in the ER membrane. The cytoplasmic domain bears both RNase and protein kinase subdomains. Point mutations used in this study debilitate the nucleotide binding site, kinase catalytic and RNase domains are indicated. **(B)** Leaf protoplasts from WT and *ire1a ire1b* mutant expressing GFP-ATG8e alone, or in *ire1a ire1b* mutant background and co-expressed with *IRE1b* constructs bearing various mutations described in **(A)**. Protoplasts were treated with 5 µg/mL TM for 6 h in the dark then imaged by confocal microscopy. Bar =10 µm. **(C)** Quantification of the number of successfully transformed protoplasts with active autophagy, defined as more than 3 autophagosomes per protoplast, was performed using epifluorescence microscopy after treating the samples for 6h with5 µg/mL TM. Three replicates with 100 protoplasts in each replicate were analyzed. Error bars represent SE. **(D)** RT-PCR analysis of the expression of *BiP3* and *bZIP60* mRNA splicing after Tm treatment, in WT and *ire1 ire1b* protoplasts, or in *ire1a ire1b* protoplasts transfected with various *IRE1b* mutants.

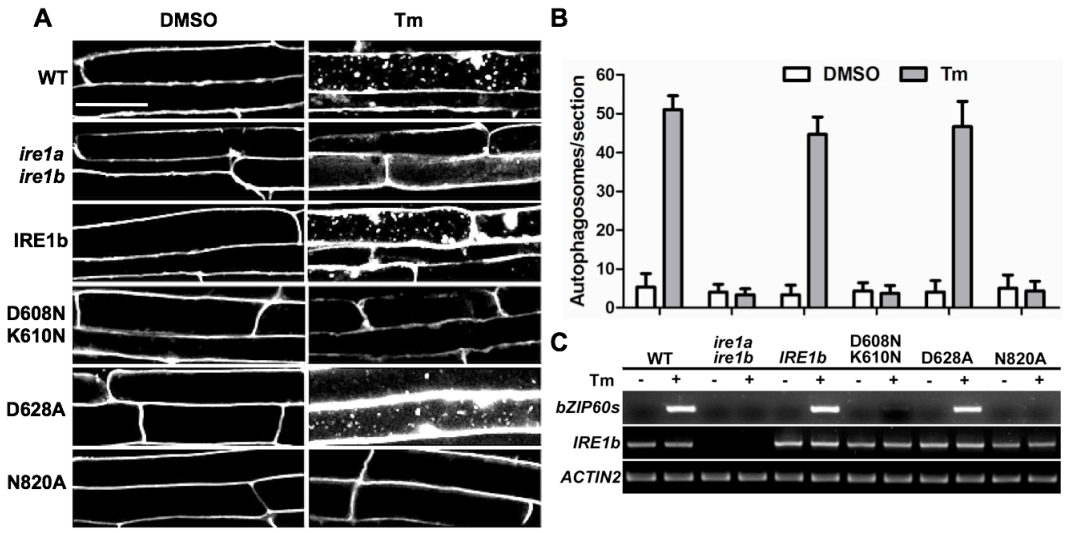

Figure 3. Complementation of defects in ER stress-induced autophagy induction in transgenic *ire1a ire1b* mutant plants bearing various *IRE1b* constructs.

(A) Seven-day-old seedlings of WT or*ire1a ire1b* expressing various *IRE1b*mutant constructs were treated in liquid 1/2 MS-0 media with 5 μg/mL TM for 6 h and then stained with MDC. Autophagosomes were visualized by confocal microscopy, bar = 50 μm. **(B)** Quantification of the number of autophagosomes in root sections was performed by fluorescence microscopy.15 sections per sample were analyzed in three biological replicates. Error bars represent SE. **(C)** Expression of *IRE1b*, *BiP3* and *bZIP60* splicing (*bZIP60s*) in the treated seedlings was analyzed by RT-PCR, and *ACTIN2* (*ACT2*) was employed as a loading control.

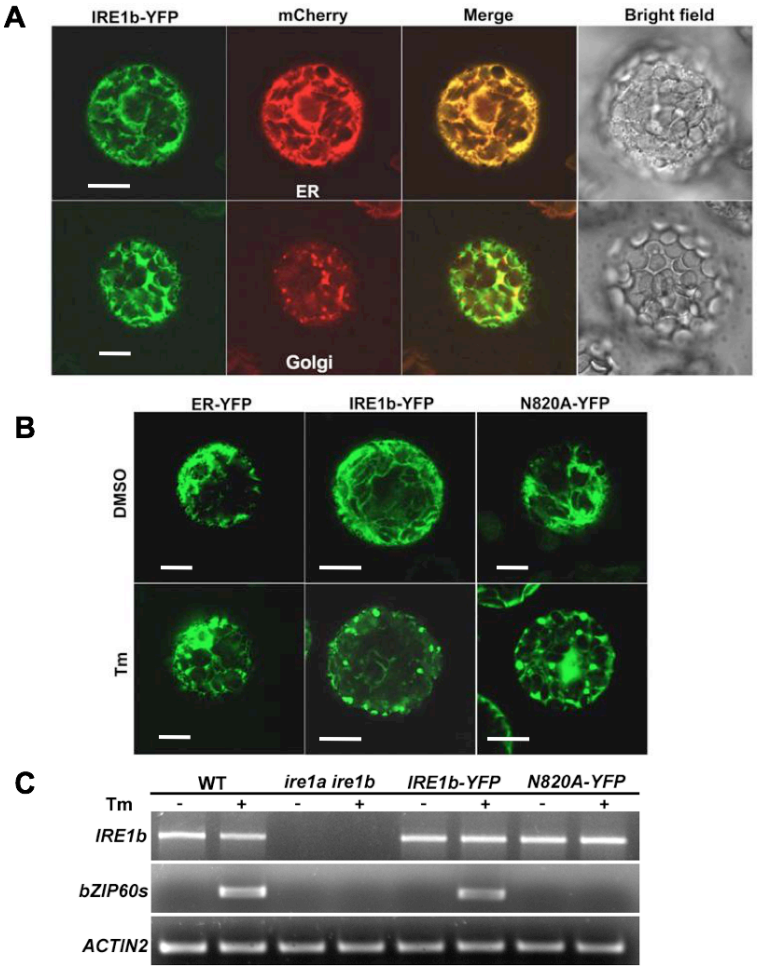

Figure 4. Clustering of IRE1b is induced upon ER stress.

(A) Co-localization of IRE1b with ER. IRE1b-YFP constructs were co-transfected with mCherry tagged ER or Golgi marker, incubated in the dark for 12 hours and subjected for observation using confocal microscopy. Golgi maker was used as a negative control. Bar =10 μm. **(B)** Confocal microscopy analysis of the clustering of IRE1b-YFP and RNase dead IRE1bN820A-YFP. Leaf protoplasts from WT seedlings were transfected with ER-YFP marker, IRE1b-YFP or RNase dead IRE1bN820A-YFP, incubated in dark for 12h, and then treated with DMSO or 5 μg/mL TM for 6h before imaging by confocal microscopy. Bar =10 μm. **(C)** *bZIP60* is spliced by *IRE1b-YFP* but not by the RNase dead *IRE1bN820A-YFP* constructs in TM treated *ire1a ire1b* protoplasts. Leaf protoplasts from WT and *ire1a ire1b* or protoplasts transfected with IRE1b-YFP or IRE1bN820A-YFP in an *ire1a ire1b* mutant background, were incubated in the dark for 12 h, treated with DMSO (as the control) or 5 μg/mL TM for 6 hours and then subjected to RNA extraction. RT-PCR was carried out to detect bZIP60 mRNA splicing (bZIP60s is the spliced form of bZIP60 mRNA), and *ACTIN2* (*ACT2*) was used as a loading control.

A

Name	Locus	*ire1a ire1b*	*ire1b*	WT	Short description
PROX-S	AT4G36430	3.52	2.81	-1.04	Peroxidase
CTS1	AT2G43620	2.48	1.72	-2.41	Chitinase
VSP1	AT5G24780	2.30	1.92	-2.50	Veg Storage Protein 1
PROX34	AT3G49120	2.20	0.95	-2.30	Peroxidase
GLH19	AT2G43610	2.09	1.10	-2.07	Glycoside hydrolase 19
PR4	AT3G04720	1.97	0.77	-2.13	PR4 protein
BGLU21	AT1G66270	1.78	0.69	-2.99	Beta glucosidase 21
PROX-P	AT2G38390	1.69	1.73	-2.41	Peroxidase
PME41	AT4G02330	1.63	0.69	-1.29	Pectinesterase
RNS2	AT2G39780	1.09	0.53	-1.00	Ribonuclease 2
PR14	AT5G01870	1.61	1.37	-1.35	Lipid transfer protein
ML	AT2G16005	1.21	1.94	-1.64	MD2-related lipid protein

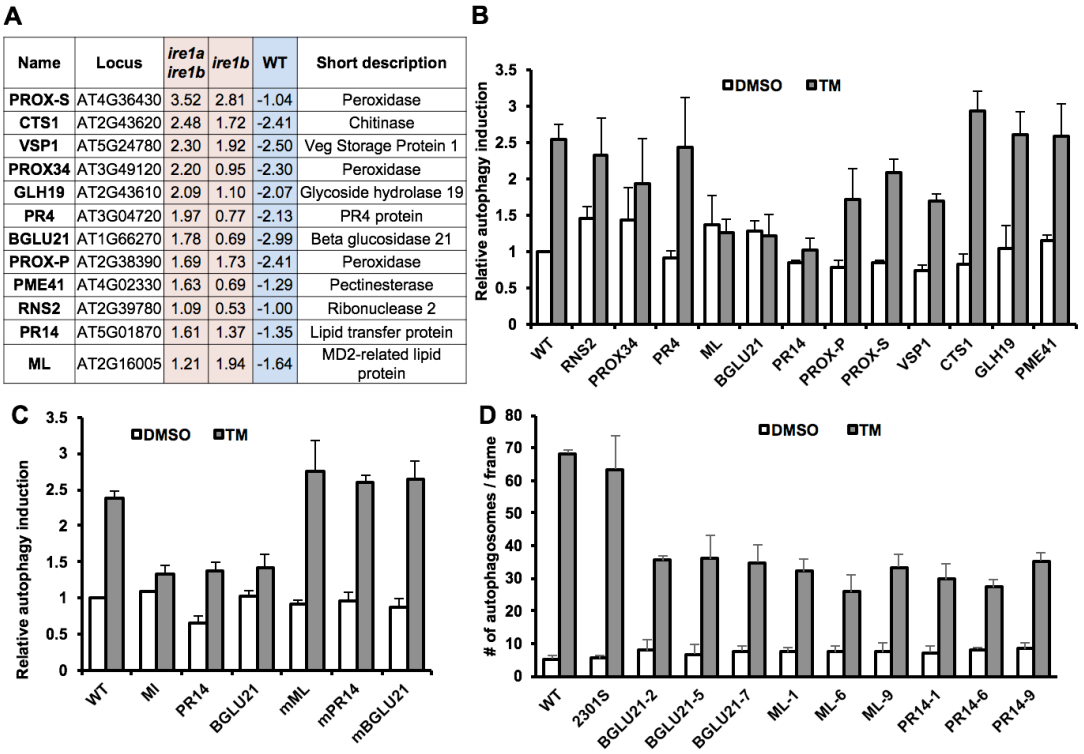

Figure 5. RIDD target genes repress ER stress induced autophagy

(A) Top 12 genes most highly downregulated in *ire1a ire1b* compared to WT, and in *ire1b* alone compared to WT. Data were obtained from the transcriptomic analysis in Supplemental Table S1. **(B)** Suppression of autophagy induction by transfecting WT leaf protoplasts with RIDD target genes. The top 12 RIDD target genes expressed from a 35S promoter were co-transfected into protoplasts with GFP-ATG8e. After incubation in the dark for 12 hours, samples were treated with DMSO (as the control) or 5 µg/mL TM for 6 hours. Autophagosomes were quantified and normalized to WT protoplasts transfected with the empty vector with DMSO treatment. The average of three replicates is shown and error bars represent SE. **(C)** Inhibition of RIDD target genes translation results in failure of autophagy suppression. *ML*, *PR14* and *BGLU21* with a frameshift mutation were expressed from a 35S promoter and co-transfected into protoplasts with GFP-ATG8e. Protoplasts were treated and examined as in **(B)**. **(D)** *ML*, *PR14* and *BGLU21* with 35S promoter were transferred into WT background, with 3 lines of each gene examined. Seven-day-old seedlings of T2 generation were transferred to liquid 1/2 MS-0 media with DMSO or 5 µg/mL TM for 6 h and then stained with MDC, followed by fluorescence microscopy. The number of autophagosomes in root sections was quantified with 10 images per sample and averaged from three biological replicates. 2301S indicates the control with empty vector transferred. Error bars represent SE.

CHAPTER 5

CONCLUSIONS AND FUTURE WORK

5.1 General conclusions and discussion

This dissertation summarized my work in investigating the functions and regulation of autophagy in plant growth and stress responses. These studies revealed that plants activate autophagy in response to abiotic stresses through TOR-dependent and –independent pathways, and identified interactions between the TOR signaling pathways and plant hormonal signaling pathways including auxin and brassinosteroids (BRs). This work also revealed the mechanism of IRE1 regulated autophagy upon ER stress (Figure 1). However, questions remain for further investigation on the mechanism of the TOR signaling pathway, the TOR-independent pathway, stress sensing for autophagy induction, and interactions between autophagy and plant hormone signaling (Figure 1).

Regulation of autophagy through the TOR-dependent pathway

My results revealed that TOR is involved in regulation of autophagy upon nutrient deficiency, salt and osmotic stress, but is not required in autophagy induced by oxidative stress or ER stress. TOR has been characterized previously as a negative regulator of autophagy in all eukaryotes and is involved in nutrient sensing (Dobrenel, Caldana et al. 2016). Autophagy is induced during senescence and nutrient deficiency (Doelling, Walker et al. 2002, Hanaoka, Noda et al. 2002), salt and drought stresses (Liu, Xiong et al. 2009), oxidative stress (Xiong, Contento et al. 2007), and ER stress (Liu, Burgos et al. 2012). However, it was unknown whether TOR is also involved in regulation of autophagy induced

upon stresses other than nutrient stress. My results showed that enhanced TOR signaling through overexpression of *TOR* or activation upon exogenous auxin IAA resulted in inhibition of autophagy induction upon both carbon and nitrogen deficiency, salt and osmotic stress, suggesting these stresses induces autophagy through a TOR-dependent pathway. However, enhanced TOR signaling failed to repress autophagy under oxidative stress or ER stress conditions, suggesting activation of autophagy upon these stresses is independent of TOR.

Although I showed that TOR is involved in autophagy regulation upon nutrient deficiency, salt and drought stress, how plants sense the stress signals and transduce to TOR is unclear. Multiple upstream regulators of TOR have been identified in yeast and mammals, but few of them are conserved in plants. One of the conserved upstream regulator of TOR is AMPK, which is a nutrient and energy sensor in mammals. AMPK negatively regulates TOR in mammals, and homologs of AMPK in yeast, Snf1, and plants, SnRK1, have been identified (Hulsmans, Rodriguez et al. 2016). SnRK1 is activated in plants upon abiotic stresses including starvation and salt stress, and interferes with nutrient remobilization process (Jossier, Bouly et al. 2009, Im, Cho et al. 2014), suggesting it might also regulate TOR activity to regulate plant growth and autophagy induction, and it might also be a salt and osmotic stress sensor for TOR signaling.

Regulation of autophagy through the TOR-independent pathway

My results showed that oxidative stress induced autophagy through a TOR-independent pathway. Oxidative stress is triggered when cells accumulate excessive reactive oxygen species (ROS), which leads to damage of cellular components including DNA,

proteins and organelles. Oxidative stress induces autophagy, and oxidized proteins are degraded by autophagy to protect cells from toxicity (Xiong, Contento et al. 2007). However, how oxidative stress triggers autophagy in plant cells remains unknown. One of the key regulator for antioxidant response is the glutathione reductase, which convert between oxidized GSSG and reduced GSH under certain conditions (Noctor and Foyer 1998). Studies in yeast and animals suggested a role of glutathione in regulation of autophagy (Desideri, Filomeni et al. 2012, Filomeni, Desideri et al. 2014), although the mechanism of how glutathione regulates autophagy remains poorly studied. It remains unclear whether plants have the same mechanism for autophagy regulation under oxidative stress, and further investigation is necessary to answer this question.

My results also revealed the mechanism of a TOR-independent pathway for regulation of autophagy upon ER stress, that is the mRNA degradation through IRE1b. As addressed earlier, ER stress induced autophagy is independent of TOR. Previous studies suggested that one isoform of IRE1, IRE1b, is required for ER stress-induced autophagy specifically (Liu, Burgos et al. 2012). IRE1 is a multifunction protein that is involved in splicing of bZIP60 mRNA for activation of unfolded protein response, mRNA degradation through the ribonuclease domain, and phosphorylation of substrates through the kinase domain. Our results revealed that the ribonuclease domain of IRE1b is required for regulation of autophagy upon ER stress. We have identified genes whose mRNA are degraded by IRE1b through RNA-seq analysis, and three of them were suggested to be negative regulators of autophagy upon ER stress. However, the mechanism of how these newly identified genes regulate autophagy is unknown, and future investigation is needed to study the IRE1b-dependent autophagy regulation upon ER stress.

Interaction between TOR regulated autophagy and plant hormone signaling pathways

Auxin has been suggested to increase TOR activity, and recent studies identified that auxin increase TOR activity through a small GTPase ROP2 (Schepetilnikov, Dimitrova et al. 2013, Li, Cai et al. 2017, Schepetilnikov, Makarian et al. 2017). Our results showed that autophagy induced by nutrient deficiency, salt and drought stress is repressed by IAA, suggesting auxin regulates autophagy with a TOR-dependent pathway, and auxin might be involved in stress tolerance through regulation of autophagy. However, whether ROP2 is required for TOR activation by auxin for autophagy regulation is unclear. Further studies are required to characterize the mechanism of auxin signaling through ROP2 for TOR activation.

We have also revealed a new interaction between brassinosteroids (BRs) and TOR signaling pathway in regulation of plant growth and autophagy upon stress responses together. Plants have evolved a sophistic network of hormonal signaling pathways in balancing growth and stress tolerance (Verma, Ravindran et al. 2016), and brassinosteroids are a family of plant hormones that is essential for plant growth and development (Li and Chory 1997). In contrast to recent studies suggesting TOR acts upstream of BR signaling for regulation of plant growth (Xiong, Zhang et al. 2016, Zhang, Zhu et al. 2016), our results showed BRs positively regulate TOR through BIN2 kinase to regulate autophagy induction. Disruption of BR signaling through chemical treatment induced autophagy, and defective BR signaling mutants displayed constitutive autophagy, whereas enhanced BR signaling led to repression of autophagy upon starvation. Furthermore, *in vitro* protein analysis revealed interaction and phosphorylation of TOR by BIN2, and potential phosphorylation sites have been identified through mass-spectrometry. Our findings therefore revealed a new function of TOR and autophagy in balancing plant growth and stress tolerance with the BR signaling

pathway. However, further investigation is needed to characterize how BIN2 inhibit TOR activity through phosphorylation, and whether plants have evolved a feedback mechanism for the interaction between BR and TOR signaling.

Our results revealed TOR as a key player in regulation of plant growth and stress tolerance with plant hormone signaling pathways including auxin and BR. However, whether TOR interacts with other hormone signaling pathways remains under studied. A recent study found a mutant *trin1* using EMS mutagenesis for TOR inhibitor resistance, suggesting TRIN1/ABI4 is a downstream effector of TOR, and it might mediate the interaction between ABA and TOR signaling (Li, Song et al. 2015). ABA signaling has been well studied for its function in plant stress tolerance and regulation of growth with complicated interactions with multiple hormone and nutrient signaling pathways (Chaiwanon, Wang et al. 2016). In another study, expression profiling through RNA-seq analysis upon inhibition of TOR also revealed a number of hormonal signaling regulated genes with differential expression, suggesting TOR might be involved in multiple hormonal signaling pathways (Dong, Xiong et al. 2015). This indicates a fruitful area of future research on the function and mechanism of TOR signaling interacting with other plant hormonal pathways.

Apart from regulation through interaction with TOR signaling, plant hormone signaling pathways might also be regulated through autophagy, particularly selective autophagy. A recent study has suggested the BR signaling transcription factor BZR1 is regulated through autophagy degradation (Zhang, Zhu et al. 2016), although the mechanism was not clear. Another BR signaling transcription factor BES1, the homolog of BZR1, have been found to be degraded by selective autophagy through interaction of the autophagosome formation protein ATG8 (Nolan, Brennan et al. 2017). ATG8 is essential for recruiting

cellular materials to autophagosomes for degradation through selective autophagy (Noda, Ohsumi et al. 2010). Evidences of protein degradation through selective autophagy are accumulating (Toyooka, Moriyasu et al. 2006, Floyd, Morriss et al. 2012). We recently observed that BIN2 has a high percentage of co-localization with ATG8 (data now shown), suggesting BR signaling might also be regulated through selective autophagy degradation of BIN2 apart from interaction with TOR, suggesting a negative feedback mechanism for BR regulated autophagy.

5.2 Future work

Characterization of the TOR signaling pathway

Although we have identified that TOR negatively regulates autophagy upon a subset of abiotic stresses, future work is needed to further characterize the function and mechanism of TOR signaling pathway under certain conditions. One question remains to be ask is that, what are the upstream regulators of TOR under different conditions? To answer this question, TOR kinase activity under different condition and in mutants of candidate upstream regulators, such as SnRK1, could be examined, through a kinase activity assay of TOR. Previous studies have been using the phosphorylation of the TOR substrate, S6K, as a marker of TOR kinase activity (Xiong, McCormack et al. 2013, Li, Cai et al. 2017). The substrate of S6K, RPS6, has also been used to indicate TOR activity (Dobrenel, Mancera-Martínez et al. 2016). For detection of TOR kinase activity, proteins extracted from plant materials after stress treatment would be run on SDS-PAGE gels, followed by western blotting and immunodetection using S6K and phospho-S6K antibodies. The ratio between phosphorylated to unphosphorylated S6K can be used to indicate the activity of TOR.

The TOR kinase activity assay will then be used to detect TOR activity under different conditions and in mutants to identify upstream stress sensors of TOR. SnRK1 has been suggested to be a positive regulator of autophagy through negative regulation of TOR in plants. To confirm SnRK1 regulates TOR upon certain stress conditions, TOR activity will be tested in SnRK1 mutants under different conditions. Activity of TOR can also be tested in different stress conditions to indicate the role of SnRK1 in sensing different stress signals.

Another question needs to be address is how TOR regulates downstream effectors for autophagy regulation. Although S6K has been confirmed and has been used as a substrate of TOR, no evidence has indicated its role in regulation of autophagy. Another candidate of TOR substrates would be the ATG1/ATG13 complex, which has been shown as a regulator of autophagy (Suttangkakul, Li et al. 2011). However, whether ATG1/ATG13 is phosphorylated and regulated by TOR in plants remains unclear. To test whether ATG1/ATG13 complex is regulated by TOR for autophagy regulation in plants, firstly protein-protein interaction assays such as GST-pull down would be used to confirm the interaction between ATG1/ATG13 and TOR in Arabidopsis both *in vitro* and *in vivo*. Constructs of *ATG1* and *ATG13* tagged with HA or other common tags would be generated for pull down assay with GST tagged *TOR* fragments. *TOR* fragments, *ATG1* and *ATG13* will also be cloned into BiFC vector to confirm its interaction with TOR *in vivo*. After confirmation of protein interaction, phosphorylation of ATG1/ATG13 would be detected through western with antibody against the tags. Phosphorylated ATG1/ATG13 might have a visible shift to distinguish between the phosphorylated form and unphosphorylated form, otherwise, proteins will be separated and detected using Phos-tag gels. If ATG1/ATG13 is

confirmed to be phosphorylated by TOR, this could also be an alternative method for detection of TOR kinase activity.

Characterization of BR and TOR signaling regulated plant growth and stress responses

Our results have suggested that TOR is involved in BR signaling in regulation of plant growth and stress response through BIN2 phosphorylation on TOR. However, it is still unclear how BIN2 regulates TOR to control the plant growth and stress response. To address this question, first we would confirm the phosphorylation of TOR by BIN2 by *in vivo* assays. Firstly, fragments of *TOR* will be cloned into constructs for Bimolecular fluorescence complementation (BiFC) assay and infiltrate with *BIN2* in BiFC constructs into tobacco for transient expression, followed by fluorescence microscopy examination of protein-protein interaction. Secondly, full length of *TOR* with an HA tag will be transiently expressed with YFP-BIN2 in tobacco, followed by detection of protein-protein interaction by immunoprecipitation. After we confirmed that TOR and BIN2 interact *in vivo* through these assays, we would also detect phosphorylation of full length TOR through kinase assay.

After confirmation of phosphorylation of TOR by BIN2 *in vivo*, we would confirm the putative phosphorylation sites identified by mass-spectrometry through targeted mutagenesis. Putative phosphorylation sites (S/T) on *TOR* fragment 3 will be mutated to D for phospho-mimic mutation or A for phospho-dead mutation, and phosphorylation of mutated *TOR* fragments will be analyzed through BIN2 kinase assay both *in vitro* and *in vivo* to confirm the specific phosphorylation residues on TOR. TOR knockout mutant is embryo lethal (Menand, Desnos et al. 2002), and hence mutated TOR fragments will be transformed to heterozygous mutant of *tor* for detection of phosphorylation and phenotypic analysis.

To further characterize how BR and TOR signaling interacts for functions in growth and stress response, we would examine global translational activity in BR and TOR mutants under certain conditions through polysome profiling and phosphoproteomics. BL would be used to induce BR signaling in TOR signaling defective mutant *raptor1b*, and AZD8055 would be used to inhibit TOR activity in BR signaling defective mutant *bin2-1D*. Samples would be compared to mutants without treatment and WT with or without treatment for global proteomics analysis. Through this experiment, we would expect to identify gene candidates that are downstream effector of the BR and TOR signaling interaction, and would have further understanding of how BR and TOR signaling interact to balance stress response and plant growth.

5.3 References

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5.4 Figures

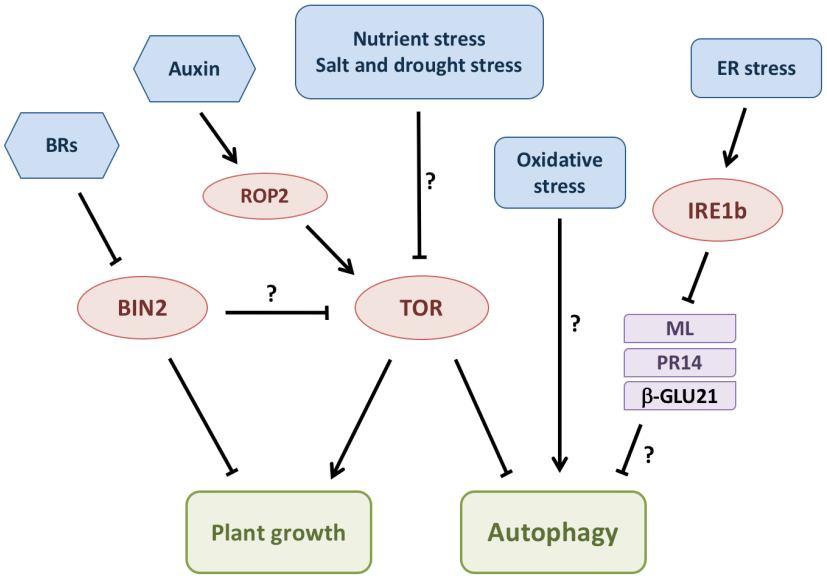

Figure 1. Schematic model and future directions of interactions between autophagy regulation, stress responses and plant growth.

TOR negatively regulates autophagy upon nutrient deficiency, salt and drought stress. Oxidative stress induces autophagy in a TOR-independent pathway that remains unknown. ER stress induces autophagy through an IRE1b dependent pathway. IRE1b is involved in mRNA degradation of ML, PR14 and β-GLU21, which are suggested to be negative regulators of autophagy, although the mechanism remains unclear. Auxin has been shown to actives TOR through ROP2 phosphorylation, and hence negatively regulate autophagy in plants. Brassinosteroids (BRs) negatively regulates BIN2 kinase activity, and eventually positively regulates plant growth. BIN2 negatively regulates TOR through phosphorylation, and hence positively regulates autophagy. However, the mechanism of inhibition of TOR by BIN2 remains to be established.