A tale of regulation: Analysis of the role of SnRK1 and glutathione in autophagy in *Arabidopsis thaliana*

by

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DEDICATION

This dissertation is dedicated to my family for their unconditional love and support over the years and for having faith on me. To my parents, for always believe that I was born to accomplish wonderful things. To my sister, for always being there for me when I needed someone just to listen. To my amazing husband Johed, for his unconditional support, encouragement, and for loving me with all his heart. To my beautiful boys, Joheniel and Javier, for inspiring me to be my best every day and making my life complete.

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ABSTRACT

Autophagy is a degradation process in which cells break down and recycle their cytoplasmic contents when subjected to environmental stress or during development. Upon activation of autophagy, a double membrane vesicle called the autophagosome forms around the cargo and delivers it to the vacuole/lysosome for degradation. In mammals, regulation of autophagy has been extensively studied. In plants, key regulators of autophagy have been identified, but their upstream components are poorly understood. This dissertation summarizes my efforts in studying the regulation of autophagy in *Arabidopsis thaliana*.

AMPK in animals, and its yeast homolog Snf1, are positive regulators of autophagy. The SnRK1 complex is the plant ortholog of AMPK and is a protein kinase that senses changes in energy levels and triggers downstream responses to enable survival. Here I demonstrate that SnRK1 is a positive regulator of autophagy in plants. Overexpression of the SnRK1 catalytic subunit, KIN10, led to increased autophagy under nutrient rich conditions, indicating activation of autophagy by SnRK1. A *kin10* mutant had a basal level of autophagy under control conditions similar to wild-type plants, but activation of autophagy by most abiotic stresses was blocked, indicating that SnRK1 is required for autophagy induction by a wide variety of stress conditions. In addition, epistasis analysis showed that SnRK1 is upstream of TOR, a negative regulator of autophagy.

Glutathione is an antioxidant that serves as scavenger of reactive oxygen species to maintain cellular homeostasis. In mammals, glutathione has been linked to activation of autophagy, but in plants this has not been reported. I demonstrate that glutathione is

required for the activation of autophagy during salt stress or nutrient starvation, but not during osmotic or oxidative stress. Furthermore, glutathione acts as a signal molecule to induce autophagy independent of reactive oxygen species. In addition, regulation of autophagy by glutathione acts upstream of TOR, most likely by regulating SnRK1 activity.

The RNS2 ribonuclease and autophagy participate in ribosomal turnover in *Arabidopsis*. Plants without RNS2 activity have constitutive autophagy. A chemical approach was used to test the SnRK1 complex and the TOR signaling pathway as possible regulators for the activation of the constitutive autophagy of *rns2-2* mutant. Here I report that activation of the TOR signaling pathway represses the constitutive autophagy in *rns2-2* mutant. Inhibition of the SnRK1 complex by trehalose-6-phosphate did not inhibit the constitutive autophagy in *rns2-2* mutant, indicating that the activation of autophagy is independent of the SnRK1 complex. Activation of TOR kinase by auxin or brassinolide resulted in the inhibition of the autophagy activity in *rns2-2* mutant, indicating that the TOR signaling pathway is involved in the activation of autophagy in *rns2-2* mutant.

In summary, this dissertation demonstrates that upon salt stress and nutrient starvation, glutathione positively regulates autophagy, most likely through activation of SnRK1, a positive autophagy regulator upstream of TOR. The TOR signaling pathway is involved in the activation of autophagy in the *rns2-2* mutant, possibly due to the inactivation of the TOR kinase.

CHAPTER 1. GENERAL INTRODUCTION: AUTOPHAGY IN PLANT CELLS AND IT'S REGULATION

A combination of sections taken from:

A review published by *Plant Physiology*[a]

Junmarie Soto-Burgos[1], Xiaohong Zhuang[2], Liwen Jiang[2], and Diane C. Bassham[1]

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1.1 Introduction

Autophagy, literally defined as “self-eating,” functions as a degradation process by recycling cytoplasmic contents under stress conditions or during development. Upon activation of autophagy, a membrane structure known as a phagophore forms and expands, finally closing to form a double-membrane vesicle called an autophagosome (Lamb et al., 2013; Yin et al., 2016). The completed autophagosome, which contains the autophagic cargo, is delivered to the vacuole (plants and yeast) or lysosome (animals). The outer membrane fuses with the vacuolar/lysosomal membrane, and the inner membrane and contents are released into the vacuole/lysosome as an autophagic body and are degraded by hydrolases. The breakdown products are transported back into the cytoplasm for reuse by the cell (Yang and Bassham, 2015) (Figure 1).

The initial identification of many autophagy-related (ATG) genes in yeast (Tsukada and Ohsumi, 1993; Thumm et al., 1994; Harding et al., 1995) was key in beginning to understand the mechanism by which autophagy occurs. The core machinery for autophagosome formation includes ATG1, which forms a complex with ATG13 for the induction of autophagy (Kamada et al., 2000); two ubiquitin-like conjugates, ATG12-ATG5 and ATG8-PE, which are recruited to the phagophore assembly site and play an important role in autophagosome formation (Yin et al., 2016); and ATG9, which may function in the recruitment of other ATG components and membrane to the forming autophagosome (Reggiori et al., 2005). In plants, autophagy has been well studied as a response to stress conditions, including nutrient deficiency (Doelling et al., 2002; Hanaoka et al., 2002), salt and drought stress (Liu et al., 2009), heat stress (Zhou et al., 2013; Yin et al., 2016),

oxidative stress (Xiong et al., 2007), hypoxia (Chen et al., 2015), pathogen attack (Liu et al., 2005; Lai et al., 2011), and endoplasmic reticulum (ER) stress (Liu et al., 2012).

1.2 Molecular Mechanisms of Autophagy

While several types of autophagy have been described morphologically in plants (Bassham et al., 2006), significant molecular information is available only for macroautophagy (hereafter autophagy), which I will discuss here. The autophagy pathway can be divided into several steps: induction, autophagosome formation, cargo selection and packaging, vesicle fusion, and breakdown (Yang and Klionsky, 2009).

1.2.1 Induction

There are several complexes involved in the induction of autophagy, the target of rapamycin (TOR) kinase complex (Noda and Ohsumi, 1998), phosphatidylinositol 3-kinase (PI3K) complex (Kihara et al., 2001), ATG1 complex (Kamada et al., 2000), and SnRK1 complex (Chen et al., 2017; Soto-Burgos and Bassham, 2017). Control of autophagy will be discussed in the section on regulation.

1.2.2 Autophagosome formation

Autophagosomes are formed from the expansion of a membrane core, termed a phagophore. The phagophore assembly site (PAS) is the proposed site for autophagosome initiation (Suzuki et al., 2001) and is defined in yeast as the place where almost all of the ATG proteins transiently colocalize (Suzuki et al., 2013). The phagophore arises from an omega-shaped structure on an ER subdomain, called the omegasome (Axe et al., 2008; Le

Bars et al., 2014). Autophagosome formation requires the recruitment of ATG1 and PI3K complexes to the omegasome, leading to phosphatidylinositol 3-phosphate (PI3P) production. PI3P is crucial for autophagosome initiation, as treatment with PI3K inhibitor completely blocked autophagosome formation (Zhuang et al., 2013; Le Bars et al., 2014).

Autophagosome formation also requires the recruitment of two conjugates, ATG12-ATG5 and ATG8-PE, to the PAS (Lamb et al., 2013). ATG12-ATG5 and ATG8-PE are generated by ubiquitin-like conjugation systems described below. Both conjugation systems have been reconstituted *in vitro* in *Arabidopsis* (Fujioka et al., 2008). The ATG9 cycling system, PI3K complex, and ATG1 complex have also been implicated in autophagosome biogenesis (Mizushima et al., 2011).

1.2.2.1 Phagophore assembly site

Fine mapping of ATG protein localization during autophagosome formation suggested that ATG proteins have individual roles and distinct locations during autophagosome expansion (Suzuki et al., 2013). The origin of the double membrane of the autophagosome is not well understood. Studies has shown that in mammals autophagosomes can form at the endoplasmic reticulum (ER)-mitochondria contact site (Hamasaki et al., 2013), and that ER exit sites as well as ER-Golgi intermediate compartments are important for this process (Hayashi-Nishino et al., 2009; Ylä-Anttila et al., 2009; Zoppino et al., 2010; Ge et al., 2013; Graef et al., 2013). In addition, it had been shown that the Golgi complex, endosomes and plasma membrane may supply lipids for autophagosome biogenesis (Ravikumar et al., 2010; van der Vaart et al., 2010; Longatti et al., 2012; Knævelsrud et al., 2013).

In plants, ATG5 has been found at the curvature domain of the phagophore at all stages and finally being detached from the phagophore once it is sealed (Le Bars et al., 2014). ATG5 has also been detected in early autophagosomal structures exhibiting a tight connection with the ER network (Le Bars et al., 2014). Unlike in animal cells, which have multiple sites at which phagophores can initiate, yeast have a single PAS, and ATG9 vesicles have been suggested to bring membrane to this location (Yamamoto et al., 2012). A recent study using advance imaging analysis showed that ATG9 have roles in the formation of early autophagosomal structures (Zhuang et al., 2017). The *atg9* mutant have dynamic extending tubules labeled with YPG-ATG8e upon dithiothreitol (DTT) and benzothiadiazole treatments. These abnormal autophagy structures had direct contact with the ER membrane, providing evidence for the ER origin of autophagosomes in *Arabidopsis*. This therefore gives insight into one of the possible membrane sources and initiation sites for autophagosome formation. Overall, it seems that during autophagy new double-membrane vesicles form *de novo* with multiple membrane sources, rather than budding from pre-existing organelles (Kim et al., 2002).

The integral membrane protein ATG9 is recruited to the PAS with the help of a PI3K complex at initial stages of phagophore formation and may play a role in the nucleation and recruitment of other ATG components such as the ATG12-ATG5-ATG16 complex and ATG8-PE adduct (Suzuki et al., 2001). In yeast, around three ATG9 molecules are required for one round of autophagosome formation (Yamamoto et al., 2012). Another important complex in ATG9 cycling and autophagosome formation is the ATG18-ATG2 complex. ATG18 binds to both ATG2 and to PI3P generated at the PAS, thus recruiting it to the PAS (Rieter et al., 2013). Interaction data shows that ATG9 is associated with ATG and non-ATG

regulators for phagophore initiation (Karanasios et al., 2016; Rao et al., 2016). In *Arabidopsis*, ATG18 is also trapped on extending tubules in *atg9* mutants, suggesting that in plants, ATG9 acts as a carrier to recycle regulators from newly formed phagophore, controlling the elongation of autophagosomal membrane (Zhuang et al., 2017).

The BAR-Domain Protein, SH3P2, has a role in autophagosome formation in *Arabidopsis*. This protein is associated with the PI3K complex and interacts with ATG8. It is localized to the PAS and actively participates in fusion events during the formation of autophagosomes. (Zhuang et al., 2013).

In yeast and mammalian cells, the cytoskeleton may drive the membrane shaping during autophagosome formation (Kast and Dominguez, 2017). Colocalization of the autophagy markers ATG8 and JOKA2 with cytoskeletal components have provided evidence of links between the cytoskeleton and plant autophagy (Ketelaar et al., 2004; Zientara-Rytter and Sirko, 2014). A mutation in the subunit of the exocyst complex, *exo70B1*, showed decreased amounts of intravacuolar autophagic bodies (Kulich et al., 2013), while the exocyst complex has been implicated to function in coordination of vesicle trafficking with the cytoskeleton (Synek et al., 2014), suggesting that EXO70B1 has a role in autophagic membrane to the vacuole. In addition, disruption of the microtubule cytoskeleton compromised autophagosome formation upon autophagic induction (Wang et al., 2015). Autophagosome formation is reduced in *nap1* mutant, a component of the SCAR/WAVE complex (Wang et al., 2016). NAP1 is initially associated with the ER and coaligns with the cytoskeleton, but when treated with constant pressure, NAP1-labeled punctae are induced and colocalize with an autophagosome marker. It is proposed that ER-associated NAP1 may

activate actin polymerization to promote membrane deformation for phagophore formation and expansion (Wang et al., 2016).

1.2.2.2 ATG12-ATG5 conjugation system

ATG12 and ATG5 are covalently and irreversibly linked through an isopeptide bond between the C-terminal glycine of ATG12 and an internal lysine residue of ATG5 (Mizushima et al., 1998) (Figure 2). Two enzymes, ATG7 and ATG10, are involved in this ubiquitination-related conjugation system. ATG7, acting as a ubiquitin-activating-like enzyme (E1), activates ATG12 by the hydrolysis of ATP, resulting in a thioester bond between the C-terminal glycine of ATG12 and a cysteine residue of ATG7 (Tanida et al., 1999; Tanida et al., 2001). The C-terminal glycine of ATG12 is transferred to a cysteine of ATG10, which functions as a ubiquitin conjugating-like enzyme (E2) and a new thioester bond is formed, releasing ATG7 (Shintani et al., 1999). An isopeptide bond is then formed between the ATG12 C-terminal glycine and the amino group of a lysine in ATG5, releasing ATG10, and producing the ATG12-ATG5 conjugate. This conjugation is essential to the formation of autophagosomes (Mizushima et al., 1999). The additional autophagy protein ATG16 self-oligomerizes and interacts with ATG5 within the ATG12-ATG5 conjugate, forming a tetrameric complex (Kuma et al., 2002).

1.2.2.3 ATG8-PE conjugation system

During autophagosome formation, ATG8 is conjugated to PE, a membrane lipid (Figure 2). In this process three enzymes are involved: ATG4, ATG7, and ATG3. ATG4, a cysteine protease, first removes the C-terminus of ATG8, leaving a glycine exposed at the C-terminus (Kirisako et al., 2000). ATG7, an E1-like enzyme, activates ATG8 by covalently

linking the exposed glycine of ATG8 to an active cysteine in ATG7. Once ATG8 is activated, it is transferred to an active cysteine residue in ATG3 through an E2-like mechanism (Ichimura et al., 2000). ATG8 is then conjugated to a PE adduct and recruited to the autophagosome membrane (Ichimura et al., 2004). The ATG12-ATG5-ATG16 complex has been suggested to function as an E3 enzyme and catalyze the conjugation step (Mizushima et al., 2002; Hanada et al., 2007; Noda et al., 2013). Unlike ATG12-ATG5 conjugation, the lipidation of ATG8 is reversible. The ATG4 protease can also deconjugate the ATG8-PE complex, recycling ATG8 and thus generating a conjugation cycle. This is essential for autophagosome expansion and the normal progression of autophagy (Kirisako et al., 2000; Thompson et al., 2005; Nair et al., 2012).

1.2.3 Cargo selection

Although autophagy is generally considered to be a nonselective process, certain proteins can be delivered to the vacuole via autophagy at a rate that suggests they are selectively targeted (Onodera and Ohsumi, 2004; Floyd et al., 2012). p62 and mNBR1 are major selective autophagy receptors in mammals, targeting ubiquitin-modified proteins and protein aggregates for autophagy (Pankiv et al., 2007; Kirkin et al., 2009; Waters et al., 2009; Ponpuak et al., 2010; Matsumoto et al., 2011). A functional ortholog that appears to be a hybrid of p62 and mNBR1 has been discovered in *Arabidopsis,* termed NBR1 (Svenning et al. 2011), and in tobacco, termed Joka2 (Zientara-Rytter et al., 2011). NBR1 and Joka2 both bind to ATG8 isoforms. Additionally, NBR1 binds to ubiquitin-modified proteins for selective autophagy. A study proposed that NBR1 targets ubiquitinated protein aggregates under stress conditions, which are likely to consist of damaged or denatured proteins (Zhou et al., 2013).

1.2.4 Vesicle fusion

After completion, autophagosomes move to and fuse with the vacuole. The timing of this process is critical. Incomplete formation of the double-membrane autophagosome prior to fusion with the vacuole would result in the cargo remaining in the cytosol. In both yeast and animals, the machinery needed for vesicle fusion includes soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptor (SNARE) proteins (Moreau et al., 2013). In yeast, additional components have been shown to be required for fusion, including the vacuolar-protein-sorting (VPS)-C and Mon1-Ccz1 (Harding et al., 1995; Darsow et al., 1997; Sato et al., 1998; Fischer von Mollard and Stevens, 1999; Kim et al., 1999; Sato et al., 2000; Wang et al., 2002; Wang et al., 2003; Itakura et al., 2012). One SNARE important for autophagy is VTI1, a v-SNARE required for vacuolar trafficking. Several VTI1 homologs are present in *Arabidopsis* (Sanmartín et al., 2007). One of these homologs, VTI12, is thought to have a function during autophagy (Surpin et al., 2003).

A plant-specific ESCRT component, FYVE domain protein required for endosomal sorting 1 (FREE1) interacts with SH3P2, and an *Arabidopsis free1* mutant accumulates abnormal autophagosome-like structures, which display a higher association with the late endosome and failure in delivery of autophagosomes to the vacuole (Gao et al., 2015). It is likely that SH3P2 promotes expansion or maturation of the developing autophagosome membrane and that FREE1- SH3P2 serves as a bridge for autophagosome fusion with the endosome/vacuole. When the autophagosome finally fuses with the vacuole, the inner membrane of the autophagosome is released into the lumen of the vacuole as an autophagic body.

1.2.5 Vesicle breakdown

After release of the autophagic body into the vacuole lumen, it is degraded, and its contents recycled. For the degradation process to occur properly in yeast, the vacuole needs to be acidic and the activity of the vacuolar hydrolases Pep4 and Prb1 is required (Nakamura et al., 1997). ATG15 is a lipase that is involved in this breakdown process and seems to function in the intravacuolar lysis of the autophagic body (Epple et al., 2001; Teter et al., 2001). ATG22 acts after the degradation process is complete to mediate the efflux of amino acids back to the cytoplasm (Yang et al., 2006). In plants, it has been suggested that hydrolases are present inside the autophagic body from its formation, allowing degradation to begin before fusion with the vacuole; additional work is needed to confirm this (Takatsuka et al., 2011; van Doorn and Papini, 2013).

1.3 Autophagy and Abiotic Stress Response

In plants, autophagy is mostly studied as a response to abiotic stress. The first and most common abiotic stress shown to induce autophagy was nutrient starvation (Doelling et al., 2002; Hanaoka et al., 2002; Thompson et al., 2005; Xiong et al., 2005; Phillips et al., 2008). Autophagy-defective plants display accelerated starvation-induced chlorosis, most likely because autophagy is required for nutrient remobilization during the starvation response. Autophagy is activated by nutrient starvation via TOR signaling pathway (Pu et al., 2017). Autophagy also functions in a variety of other abiotic stresses like, oxidative stress (Xiong et al., 2007), hypoxia (Chen et al., 2015), salt and drought stress (Liu et al., 2009), heat stress (Zhou et al., 2013; Yin et al., 2016), and ER stress (Liu et al., 2012).

Oxidative stress occurs when cells accumulate excessive reactive oxygen species (ROS) and therefore cause damage to cellular components. When oxidative stress is triggered using hydrogen peroxide or methyl viologen, autophagy is induced. The autophagy-defective RNAi-*AtATG18a* plants are more sensitive to oxidative stress and accumulate higher levels of oxidized protein aggregates (Xiong et al., 2007; Xiong et al., 2007); similar phenomena have also been observed in the rice *Osatg10* mutant (Shin et al., 2009). These results suggest that autophagy plays a role in degrading oxidized proteins in plant cells.

Autophagy is also required for plant tolerance to drought and salt stresses (Liu et al., 2009), implying that autophagy plays a role in removing damaged proteins or organelles during these stresses. Both salt and drought stress lead to osmotic stress response, but salt stress also triggers ionic stress (Zhu, 2016). Autophagy is induced in response to salt and osmotic stress, and autophagy mutants are hypersensitive to both stress conditions (Liu et al., 2009), suggesting autophagy is also important for salt and drought stress tolerance.

The unfolded protein response (UPR) is activated by accumulation of unfolded and misfolded proteins within the ER during ER stress (Liu and Howell, 2016). ER stress induces autophagy and it has been shown that the ER can be degraded by autophagy (Liu et al., 2012). Autophagy deficient mutants exhibits hypersensitivity to most of the abiotic stresses mentioned before, suggesting that the activation of autophagy under these conditions is important for plant survival (Wang et al., 2017). During these conditions, the ROS production can act as a signal to activate stress responses, including autophagy. The ROS can be produced partially through NADPH oxidase, although autophagy can be regulated in a NADPH oxidase dependent and -independent manner in *Arabidopsis* (Liu et al., 2009; Chen et al., 2015).

1.4 Autophagy and Plant Development

Under normal growth conditions, a basal level of autophagy contributes to cell homeostasis (Wang et al., 2017). Autophagy defective mutants can complete their life cycles under normal growth conditions, but their leaves senesce prematurely (Doelling et al., 2002; Hanaoka et al., 2002; Yoshimoto et al., 2004; Thompson et al., 2005; Qin et al., 2007; Phillips et al., 2008), due to salicylic acid accumulation (Yoshimoto et al., 2009).

Autophagy is involved in the remobilization of nutrients, which is require for seed germination and senescence (Liu and Bassham, 2012). In *Arabidopsis*, autophagy mutants generate normal seeds under normal growth conditions, but ATG genes are differentially expressed during seeds development (Angelovici 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 et al., 2014). In maize, as seeds mature following pollination, ATG8 lipidation, an indication of autophagy pathway activity, increases in the starchy endosperm (Chung et al., 2009). Abundant lipidated ATG8 was detected after seed germination, suggesting a role for autophagy in mobilizing nutrients from the endosperm for early seedling development (Chung et al., 2009). Autophagy is also important for root cell growth and root hair formation in *Arabidopsis* (Inoue et al., 2006).

1.5 Regulation of Autophagy

In animals and yeast, many regulators of autophagy have been identified, however, some of them are not conserved or missing in plants (Michaeli et al., 2016). In plants, several components of autophagy regulation have been identified, including SnRK1 complex, TOR,

ATG1 complex and IRE1 (Figure 3). In this section, we will discuss known regulators of the autophagy pathway in plants.

1.5.1 SnRK1 activates autophagy in response to abiotic stress

Autophagy is induced by nutrient depletion, most likely as a mechanism for nutrient recycling and generation of substrates for energy metabolism (Doelling et al., 2002; Hanaoka et al., 2002). The energy sensor Snf1-related protein kinase 1 (SnRK1) is a heterotrimeric complex that has been suggested to be a master regulator of metabolism in plants in response to nutrient and energy deficiency (Sugden et al., 1999; Baena-González et al., 2007; Crozet et al., 2014). The animal and yeast orthologs of SnRK1, AMP-activated kinase (AMPK) and Suc nonfermenting 1 (Snf1), respectively, are energy and metabolic sensors that maintain cellular homeostasis and activate autophagy under low-energy conditions (Hardie, 2011; Carroll and Dunlop, 2017). AMPK/Snf1 can regulate autophagy by inhibiting the target of rapamycin (TOR) complex (Lee et al., 2010), a negative regulator of autophagy, therefore allowing autophagy to become active, or by direct phosphorylation of ATG1, which also leads to the activation of autophagy (Wang et al., 2001; Egan et al., 2011; Kim et al., 2011). In *Arabidopsis* (*Arabidopsis thaliana*), there are two isoforms of the SnRK1 complex catalytic subunit, KIN10 and KIN11, with KIN10 being responsible for most of the SnRK1 activity (Baena-González et al., 2007; Jossier et al., 2009; Crozet et al., 2014). A *kin10 kin11* double mutant is lethal, and reduced expression via virus- induced gene silencing leads to decreased activation of stress and starvation genes and to deformed leaves, flowers, and inflorescence (Baena-González et al., 2007), indicating that SnRK1 functions in development and stress responses.

Overexpression of the KIN10 gene in *Arabidopsis* leads to constitutive activation of autophagy (Chen et al., 2017; Soto-Burgos and Bassham, 2017), suggesting a positive role in the regulation of autophagy. KIN10 overexpression led to increased phosphorylation of ATG1 during sucrose starvation, suggesting that KIN10 regulates autophagy by affecting the phosphorylation of ATG1 (Chen et al., 2017), as in mammals and yeast. Autophagy is activated during a wide range of abiotic stresses, and a *kin10* knockout mutant failed to activate autophagy during most of these stresses (Soto-Burgos and Bassham, 2017). This indicates that KIN10 activates autophagy not just in response to nutrient deficiency or energy depletion as predicted, but also during other abiotic stresses, indicating a wider role for SnRK1 in regulation of autophagy than previously expected.

1.5.2 TOR is a negative regulator of autophagy

The TOR complex is a key regulator of the balance between growth and autophagy in all eukaryotes tested (Noda and Ohsumi, 1998; Pattingre et al., 2008; Liu and Bassham, 2010). In plants, TOR is activated when nutrients are abundant, in turn enhancing mRNA translation initiation, ribosome biogenesis, cell wall synthesis and growth and inhibiting autophagy (Deprost et al., 2007; Ren et al., 2011; Xiong and Sheen, 2015). Upon nutrient deficiency, TOR is inactivated, reducing growth and allowing the activation of autophagy (Pattingre et al., 2008).

The *Arabidopsis* TOR complex consists of TOR itself, a Ser/Thr kinase (Menand et al., 2002), the Regulatory Associated Protein of TOR, RAPTOR (Anderson et al., 2005; Deprost et al., 2005), which presents substrates to TOR for phosphorylation (Hara et al., 2002), and Lethal with Sec Thirteen 8, which stabilizes the complex (Moreau et al., 2012). A complete knockout of TOR is embryo-lethal (Menand et al., 2002), while knockdown by

RNA interference leads to arrest of plant growth and development and constitutive autophagy (Deprost et al., 2007; Liu and Bassham, 2010). Disruption of RAPTOR in *Arabidopsis* similarly leads to defects in plant growth and development, although less severe than those of a TOR knockout, as well as constitutive autophagy (Anderson et al., 2005; Deprost et al., 2005; Pu et al., 2017). Overexpression of TOR blocks autophagy induced by nutrient starvation, salt, and osmotic stresses, while autophagy induced by oxidative and ER stress is not affected (Pu et al., 2017). Regulation of autophagy can therefore be TOR dependent or independent (Figure 3) depending on the environmental stress to which the plant is subjected.

RAPTOR interacts with ribosomal p70 S6 kinase (S6K) in response to osmotic stress signals, suggesting a role for S6K in the TOR signaling pathway and plant stress responses (Mahfouz et al., 2006). Several other proteins have also been shown to interact with RAPTOR or TOR *in vitro*, including *Arabidopsis* Mei2-like1 (Anderson et al., 2005) and transcription factor E2Fa. Tap42/a4 is an effector of TOR in yeast and mammals, and its *Arabidopsis* homolog, Tap46, has been identified as a downstream target of TOR. Tap46 is phosphorylated by TOR and interacts with protein phosphatase type 2A, a regulator of autophagy in yeast (Ahn et al., 2011). Overexpression or reduction of expression of Tap46 correlates with TOR activity (Ahn et al., 2015), suggesting that Tap46 is a positive regulator of the TOR pathway. Silencing of the TAP46 gene using virus-induced gene silencing in tobacco (*Nicotiana tabacum*) led to induction of autophagy, as in the *TOR* RNAi plants (Ahn et al., 2011), indicating that it can negatively regulate autophagy. This suggests that Tap46 acts as a positive effector in the TOR signaling pathway, leading to the regulation of autophagy.

Recently, a connection between the TOR signaling pathway and SnRK1 complex has been demonstrated. KIN10 interacts with RAPTOR *in vivo* and can phosphorylate RAPTOR *in vitro*, like its mammalian orthologs (Nukarinen et al., 2016). Blocking TOR activity in a *kin10* mutant led to activation of autophagy, while inhibition of SnRK1 activity in a *raptor1b* mutant failed to block the constitutive autophagy observed in this mutant (Soto-Burgos and Bassham, 2017). SnRK1 therefore acts upstream of the TOR signaling pathway in the regulation of autophagy (Soto-Burgos and Bassham, 2017), at least under conditions in which activation of autophagy is TOR dependent.

1.5.3 The ATG1 kinase complex

A major regulator of autophagy and a downstream substrate of TOR in yeast and animals is the ATG1/ATG13 kinase complex. ATG1 is the catalytic subunit of the complex and activates autophagy in response to nutrient depletion (Díaz-Troya et al., 2008; Mizushima, 2010). In mammals, AMPK promotes autophagy by phosphorylating Ulk1 (ATG1 homolog) upon Glc starvation (Kim et al., 2011). In *Arabidopsis*, KIN10 overexpression results in an increase in ATG1 phosphorylation, suggesting that this mode of regulation is conserved (Chen et al., 2017). In yeast, TOR phosphorylates ATG13 in nutrient-rich conditions, causing a decrease in its affinity for ATG1, preventing their association and therefore repressing autophagy. Under starvation conditions, TOR becomes inactive, leading to the dephosphorylation of ATG13, allowing ATG1 to associate with ATG13 and activate autophagy (Nakatogawa et al., 2009; Kamada et al., 2010). In mammals, unlike in yeast, ATG1 associates with ATG13 under all conditions, indicating that the regulatory mechanism of ATG1/ ATG13 differs between mammals and yeast (Lee et al., 2007).

In *Arabidopsis*, ATG1 and ATG13 are present in multiple copies, which are most likely functionally redundant. During nutrient starvation, ATG1a is hyper-phosphorylated, potentially by SnRK1 (Chen et al., 2017), while ATG13a is hypo-phosphorylated (Suttangkakul et al., 2011), suggesting that the ATG1 complex functions in a manner similar to yeast in terms of autophagy regulation. Disruption of ATG13 leads to phenotypes similar to those of mutants in other core autophagy genes, with hypersensitivity to nutrient starvation and accelerated senescence. *atg13* mutants have defects in the formation of autophagic bodies, suggesting that the complex acts upstream of autophagosome formation (Suttangkakul et al., 2011). ATG1a associates with autophagic bodies and is delivered to the vacuole for degradation, indicating that the ATG1 complex is a substrate of autophagy. A negative feedback mechanism is therefore proposed to exist to reduce activated ATG1 complex levels after induction of autophagy by nutrient deficiency (Suttangkakul et al., 2011). This turnover might be an attempt to reset autophagic induction by requiring the incorporation of freshly activated ATG1 kinase during each round of phagophore assembly (Suttangkakul et al., 2011). Based on this evidence, and by comparison with ATG1 complex functions in animals and yeast, we hypothesize that the ATG1 complex may regulate autophagy in *Arabidopsis* via its phosphorylation by TOR and/or SnRK1 (Figure 3).

1.5.4 Regulation of autophagy by IRE1 during ER stress

Autophagy is induced by ER stress, in which accumulation of unfolded and misfolded proteins within the ER activates the unfolded protein response (UPR) (Liu et al., 2012; Liu and Howell, 2016). Although repression of TOR activity leads to activation of autophagy during some abiotic stresses, autophagy induced by ER stress seems to be independent of TOR (Pu et al., 2017), as autophagosomes are formed normally during ER stress in TOR

overexpression lines. Instead, ER stress-induced autophagy depends on inositol-requiring enzyme-1 (IRE1), an ER stress sensor that activates the UPR (Cox and Walter, 1996; Mori et al., 1996; Chen and Brandizzi, 2013). During ER stress, IRE1 is activated by oligomerization and autophosphorylation (Korennykh et al., 2009). After activation, IRE1 splices an mRNA encoding a membrane-associated basic Leu zipper transcription factor (bZIP60) (Nagashima et al., 2011). The spliced bZIP60 mRNA is translated, producing an active protein that is translocated into the nucleus and upregulates UPR genes such as BIP (Iwata and Koizumi, 2005; Deng et al., 2011).

Two IRE1 genes have been identified in *Arabidopsi*s, IRE1a and IRE1b (Koizumi et al., 2001; Deng et al., 2011; Moreno et al., 2012), and a mutant defective in IRE1b is unable to form autophagosomes after inducing ER stress using dithiothreitol (DTT) or tunicamycin, indicating that IRE1b is required for the induction of autophagy by ER stress (Liu et al., 2012). Mutations in either IRE1a or bZIP60 have no effect on autophagy during ER stress, suggesting that only IRE1b is involved in the regulation of autophagy and that its bZIP60 splicing activity is not required (Liu et al., 2012). The addition of chemical chaperones or overexpression of molecular chaperones inhibited activation of autophagy by DTT or tunicamycin, and expression of a misfolded protein mimic in the ER was sufficient to induce autophagy via IRE1b activity. The accumulation of unfolded proteins in the ER, presumably recognized by IRE1b, is therefore a key event in activating autophagy during ER stress (Yang et al., 2016).

Excessive heat is a major factor that causes ER stress, as indicated by the splicing of bZIP60 mRNA by IRE1b (Deng et al., 2011) and by upregulation of BiP (Leborgne-Castel et al., 1999). The induction of autophagy by heat stress is also mainly due to the accumulation

of misfolded proteins (Yang et al., 2016). Autophagy activation is significantly reduced in an *ire1b* mutant during heat stress, compared to the wild type, indicating that the autophagy response during heat stress is dependent on IRE1b (Yang et al., 2016) and is most likely primarily acting as an ER stress response.

1.5.5 Other possible regulators

Most of the identified regulators of plant autophagy act post-translationally, and relatively little is known about transcriptional regulation of autophagy-related genes. In tomato (*Solanum lycopersicum*), the transcription factor HsfA1a has been shown to induce drought tolerance by the activation of ATG genes and the induction of autophagy (Wang et al., 2015). The *Arabidopsis* WRKY33 transcription factor has also been suggested to regulate autophagy. WRKY33 is important for plant resistance to necrotrophic pathogens (Zheng et al., 2006), and a yeast two-hybrid screen showed that WRKY33 interacts with ATG18a, a core autophagy component (Lai et al., 2011). Furthermore, a *wrky33* mutant was defective in upregulation of ATG18a and induction of autophagy upon infection with *Botrytis cinerea* (Lai et al., 2011). Silencing of WRKY33 in tomato led to reduced ATG gene expression and autophagosome accumulation during heat stress (Zhou et al., 2014), suggesting that it also functions in abiotic stress responses.

Several new pathways for regulation of autophagy in plants have been identified recently. First, the glyceraldehyde-3-phosphate dehydrogenases (GAPDH) have been shown to negatively regulate autophagy (Han et al., 2015; Henry et al., 2015). In *Arabidopsis*, there are multiple isoforms of GADPH, including chloroplastic photosynthetic (GAPA1, GAPA2, and GAPB) and cytosolic glycolytic (GAPC1 and GAPC2) enzymes (Zaffagnini et al., 2013). Mutants in the GADPH isoforms GAPA1 and GAPC1 have constitutive autophagy,

suggesting that GAPDH can negatively regulate autophagy (Henry et al., 2015). In tobacco, silencing of GAPCs activated autophagy, whereas overexpression of GAPCs inhibited oxidative stress-induced autophagy (Han et al., 2015). Furthermore, GAPCs interact with ATG3 *in vivo* and *in vitro*, but upon oxidative stress this interaction weakens (Han et al., 2015). Disruption of GAPDHs led to enhanced disease resistance (Han et al., 2015; Henry et al., 2015), although whether this is related to autophagy is unclear. Together, these data suggest that GADPH negatively regulates autophagy through interaction with ATG3.

Second, hydrogen sulfide has been linked to the regulation of autophagy. Hydrogen sulfide is an important signaling molecule in mammalian systems, and emerging data suggest that this is also true in plants. It has been identified as a component of the ABA signaling pathway (García-Mata and Lamattina, 2010) and has roles in regulation of photosynthesis (Chen et al., 2011) and tolerance to copper (Zhang et al., 2008), aluminum (Zhang et al., 2010), and boron (Wang et al., 2010) stress. DES1 is an L-Cys desulfhydrase that is involved in the production of hydrogen sulfide and the degradation of Cys (Alvarez et al., 2010). A mutation in the *DES1* gene impedes sulfide generation in the cytosol and promotes the accumulation of ATG8 and ATG8-PE, indicating activation of autophagy. Furthermore, addition of exogenous sulfide to a *des1* mutant or genetic complementation of *DES1* gene prevented the accumulation and lipidation of ATG8 proteins (Álvarez et al., 2012). Recently, it was demonstrated that the negative regulation of autophagy by sulfide is independent of reactive oxygen species, and sulfide therefore probably regulates autophagy by an alternative pathway (Laureano-Marín et al., 2016).

Third, the plant Bax inhibitor-1 (BI-1) has recently been shown to interact with ATG6 *in vivo* and *in vitro* and to positively regulate autophagy (Xu et al., 2017). Silencing of

tobacco BI-1 reduced the autophagy activity induced by virus infection or oxidative stress, while overexpression of BI-1 increased autophagy activity and caused autophagy- dependent cell death (Xu et al., 2017). BI-1 therefore has both autophagy-dependent pro-survival and pro-death effects, depending on the conditions.

As research progresses, more information becomes available about how autophagy is regulated in plants. Although new discoveries have been made, further research is needed to fully understand how the autophagy pathway is controlled under different conditions and how the regulatory components are coordinated to determine the degree of autophagy activation.

1.6 Dissertation Organization

This dissertation summarizes my research findings on the regulation of autophagy during abiotic stress and in plants without the RNS2 ribonuclease activity in *Arabidopsis thaliana.*

Chapter 1 provides a general introduction and review of our current understanding of autophagy in plants.

Chapter 2 demonstrates that the SnRK1 complex positively regulates autophagy in *Arabidopsis* under abiotic stress. My results suggest that autophagy regulation by SnRK1 occurs upstream the TOR signaling pathway. I performed all the experiments described in this chapter and drafted the manuscript. Dr. Diane Bassham extensively edited the manuscript and provided valuable suggestions.

Chapter 3 reports the first link between glutathione and regulation of autophagy in *Arabidopsis*. My results illustrate that glutathione is required for the activation of autophagy during salt stress and sucrose starvation. Xiaoyi Liu performed the experiment shown in figure 1. Yimo Liu did the preliminary experiments that started this research project. I performed the remaining experimental work and wrote the chapter. Dr. Bassham edited the chapter and provided valuable suggestions.

Chapter 4 reports a possible mechanism by which autophagy is activated in plants lacking RNS2 ribonuclease activity. My results indicate that activation of TOR kinase can suppress the constitutive autophagy in *rns2-2* mutant. I performed all the experimental work and wrote the chapter with feedback from Dr. Bassham.

Chapter 5 summarizes the conclusions of this research and discusses implications as well as areas of future work.

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1.8 Figures

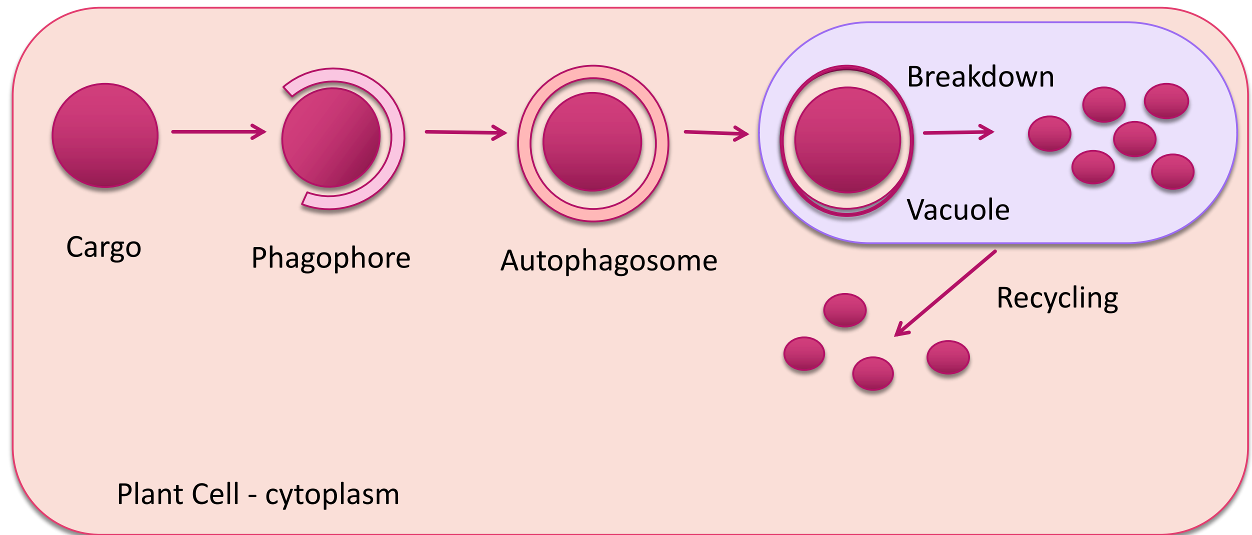

Figure 1. Pathway for autophagy in plant cells

Upon induction of autophagy, a double-membrane structure called phagophore forms around the cargo to be degraded. Upon, phagophore closure it becomes the autophagosome. The autophagosome then transports the cargo to the vacuole. During the fusion process, the outer autophagosome membrane fuses with the vacuole membrane, and the remaining single-membrane structure (termed an autophagic body) is delivered inside the vacuole. The autophagic bodies are then broken down by vacuolar hydrolases, and the products are exported from the vacuole to the cytoplasm for reuse.

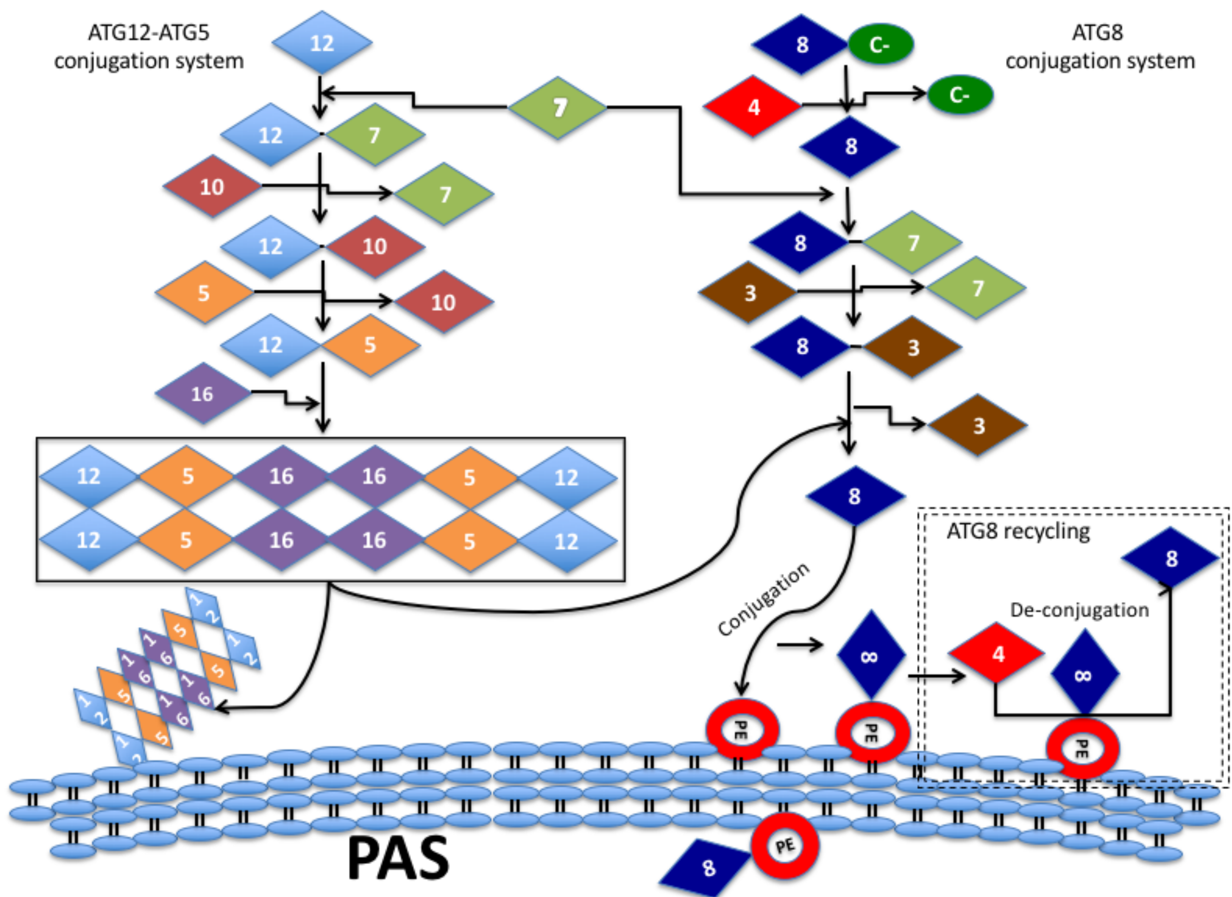

Figure 2. Two ubiquitin-like conjugation systems function in autophagosome formation.

(*Left*) The ATG12-ATG5 conjugation system is depicted. ATG12 binds to ATG7 and then is transferred to ATG10. Finally, ATG12 is covalently conjugated to ATG5 to form the ATG12-ATG5 complex. This complex interacts with ATG16 and forms a tetramer, which is recruited to the PAS. (*Right*) The ATG8 conjugation system is depicted. First the ATG4 protease cleaves the C-terminus of ATG8, and ATG8 binds to ATG7. Next, ATG8 is transferred to ATG3. Once ATG8 dissociates from ATG3 it is conjugated to phosphatidylethanolamine (PE) in the PAS. The ATG12-ATG5•ATG16 complex acts as an E3-like enzyme during the conjugation of ATG8 with PE. Since the protease ATG4 can cleave the ATG8-PE adduct, this reaction is reversible, freeing ATG8 into the cytosol. ATG proteins are depicted as diamonds with their respective number inside.

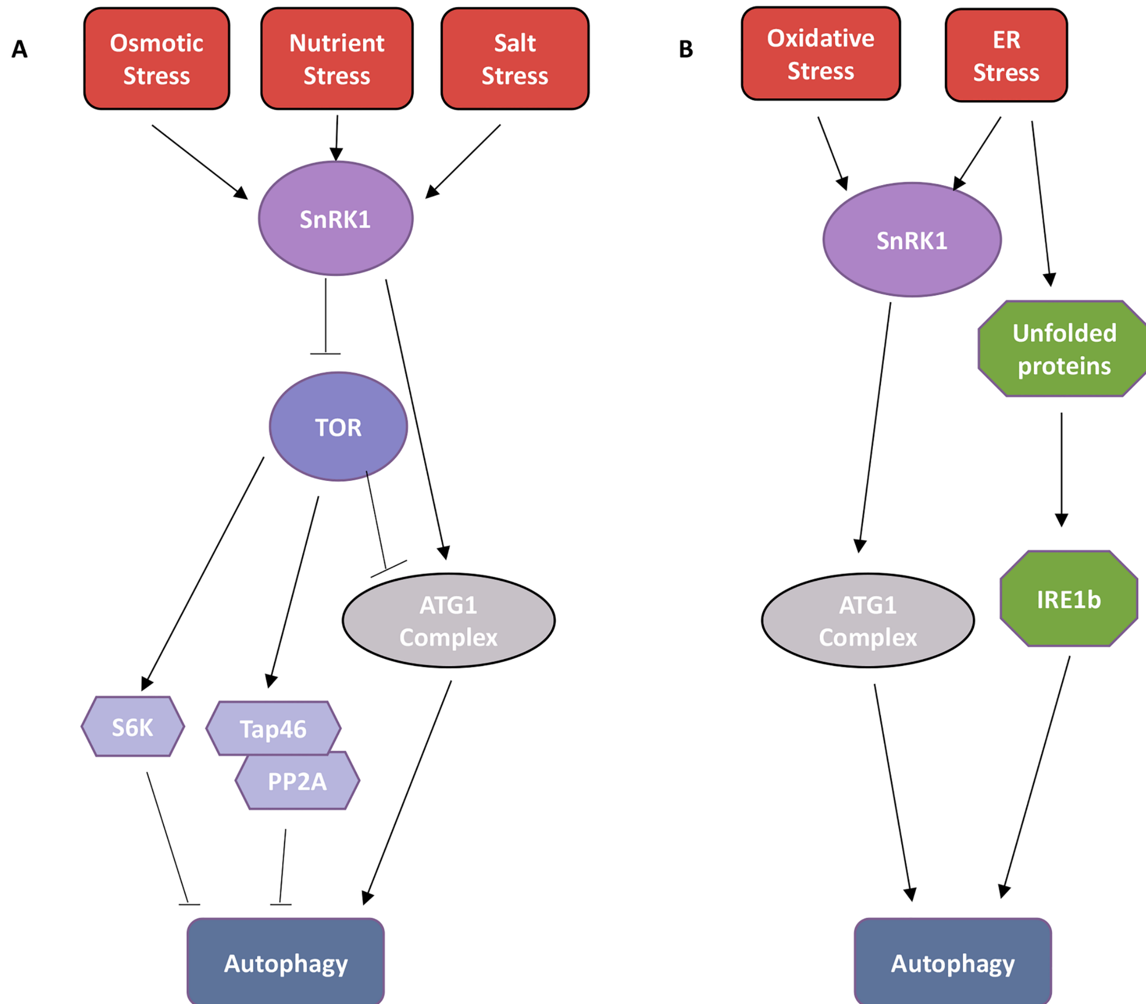

Figure 3. TOR-dependent and -independent regulatory pathways for autophagy in plants

Autophagy can be activated by abiotic stress, including osmotic, nutrient, salt, oxidative and ER stress. This activation can be regulated in a TOR-dependent or –independent manner. **(A)** Upon osmotic, nutrient or salt stress, the SnRK1 complex can inhibit TOR, leading to activation of the ATG1 complex or deactivation of S6K and Tap46, in turn activating autophagy. SnRK1 can also activate ATG1 complex by phosphorylation of ATG1, leading to activation of autophagy. **(B)** Upon oxidative or ER stress, SnRK1 activates the ATG1 complex, leading to the activation of autophagy. Upon ER stress, unfolded proteins accumulate and activate IRE1b, leading to autophagy. Ovals represent kinase complexes, hexagons represent TOR targets, and octagons represent components of ER stress response pathway.

CHAPTER 2. SnRK1 ACTIVATES AUTOPHAGY VIA THE TOR SIGNALING PATHWAY IN *Arabidopsis thaliana*

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2.1 Abstract

Autophagy is a degradation process in which cells break down and recycle their cytoplasmic contents when subjected to environmental stress or during cellular remodeling. The *Arabidopsis thaliana* SnRK1 complex is a protein kinase that senses changes in energy levels and triggers downstream responses to enable survival. Its mammalian ortholog, AMPK, and yeast ortholog, Snf-1, activate autophagy in response to low energy conditions. We therefore hypothesized that SnRK1 may play a role in the regulation of autophagy in response to nutrient or energy deficiency in *Arabidopsis*. To test this hypothesis, we determined the effect of overexpression or knockout of the SnRK1 catalytic subunit KIN10 on autophagy activation by abiotic stresses, including nutrient deficiency, salt, osmotic, oxidative, and ER stress. While wild-type plants had low basal autophagy activity in control conditions, KIN10 overexpression lines had increased autophagy under these conditions, indicating activation of autophagy by SnRK1. A *kin10* mutant had a basal level of autophagy under control conditions similar to wild-type plants, but activation of autophagy by most

abiotic stresses was blocked, indicating that SnRK1 is required for autophagy induction by a wide variety of stress conditions. In mammals, TOR is a negative regulator of autophagy, and AMPK acts to activate autophagy both upstream of TOR, by inhibiting its activity, and in a parallel pathway. Inhibition of *Arabidopsis* TOR leads to activation of autophagy; inhibition of SnRK1 did not block this activation. Furthermore, an increase in SnRK1 activity was unable to induce autophagy when TOR was also activated. These results demonstrate that SnRK1 acts upstream of TOR in the activation of autophagy in *Arabidopsis*.

2.2 Introduction

Autophagy (self-eating) is a degradation process in which cells recycle cytoplasmic contents during development or when under stress. Upon autophagy activation, cellular components are degraded in the lysosome/vacuole and the products are recycled back into the cytoplasm [1]. Macroautophagy (hereafter referred to as autophagy) is characterized by the formation of a double-membrane vesicle called an autophagosome, which delivers cargo to the vacuole for degradation. To maintain homeostasis under normal conditions, cells have a basal level of autophagy to turn over damaged proteins or organelles, and the pathway is upregulated during stress [1]. Many autophagy-related (*ATG*) genes were initially identified in yeast [2-4], followed by identification of their homologs in other organisms. Major factors include ATG1, which together with ATG13 forms a complex involved in the induction of autophagy [5]. Two ubiquitin-like conjugates, ATG12/ATG5 and ATG8-PE, are recruited to the phagophore assembly site (PAS) and play an important role in autophagosome formation [5]. ATG9 is also recruited to the PAS and may function in the recruitment of other ATG components and membrane to the forming autophagosome [6]. In plants, autophagy is

activated in response to many biotic and abiotic stresses, including pathogen attack [7, 8], nutrient deficiency [9, 10], salt, osmotic [11], endoplasmic reticulum (ER) [12], hypoxia [13] and oxidative stress [14].

A substantial number of upstream regulators of autophagy have been identified in animal cells, but the majority are not conserved in plants. One exception is the target of rapamycin (TOR), a phosphatidylinositol 3-kinase-related kinase that acts as a negative regulator of autophagy [15-17]. The central component of the TOR signaling pathway is the TOR complex, which in *Arabidopsis* consists of the serine/threonine kinase TOR itself [18], RAPTOR [19, 20], which presents substrates to TOR for phosphorylation [21], and LST8, which stabilizes the complex [22]. TOR coordinates autophagy with growth by controlling processes, for example translation initiation, that regulate growth in response to nutrient status [23, 24]. In nutrient-rich conditions, TOR is activated and inhibits autophagy, probably by phosphorylation of the ATG1 complex, which has been shown to regulate autophagy in *Arabidopsis* [25]. Upon nutrient deprivation, the TOR complex is inactivated, allowing the activation of autophagy and down-regulating growth. Consistent with this, an *Arabidopsis* knockout mutant in *TOR* is embryo-lethal [18], and lines with decreased *TOR* expression have reduced growth [23] and constitutive autophagy [17]. Conversely, TOR overexpression inhibits activation of autophagy in response to multiple abiotic stresses [26].

AMP-activated protein kinase (AMPK) in animals, and its yeast homolog sucrose non-fermenting 1 (Snf1), are positive regulators of autophagy. AMPK and Snf1 are energy and metabolic sensors that maintain cellular energy homeostasis [27, 28]. They are activated by an increase in the AMP:ATP and ADP:ATP ratios, which promotes phosphorylation of AMPK/Snf1 by upstream kinases [29-32]. Upon activation, AMPK implements an energy-

saving program by transcriptional control and enzyme regulation [32]. Catabolic pathways such as fatty acid oxidation, glycolysis and autophagy are activated, while anabolic processes, including synthesis of cholesterol, proteins and fatty acids, are switched off [33]. AMPK/Snf1 regulates autophagy via two pathways: by inhibiting the TOR complex [34], therefore allowing autophagy to become active, or by the direct phosphorylation of ATG1, also leading to activation of autophagy [35-37].

The Snf1-related protein kinase 1 (SnRK1) is a plant ortholog of AMPK and Snf1, and is a heterotrimeric complex that functions as an energy sensor [33, 38]. SnRK1 is composed of a catalytic (α) and two regulatory (β, γ) subunits [33]. The regulatory subunits β and γ can be classified into two groups: the classical subunits [β_1, β_2 and γ] (conserved with mammals and yeast) and the plant-specific subunits [β_3 and $\beta\gamma$] [32, 39, 40]. Even though the γ subunit exists in plants, the majority of SnRK1 active complexes contain the $\beta\gamma$ hybrid subunit acting as the canonical γ subunit [41]. Three isoforms of the catalytic subunit exist in *Arabidopsis*, KIN10, KIN11 and KIN12, but only KIN10 and KIN11 appear to be expressed [42]. Of these two, KIN10 is responsible for most of the SnRK1 activity [43]. KIN10 and KIN11 can act in opposition to one another in some situations, indicating that they can perform separate functions. For example, overexpression of KIN10 results in late flowering while overexpression of KIN11 causes early flowering [44]. A *kin10 kin11* double knockout mutant is lethal, suggesting that there is also some redundancy in gene function [42]. A reduction in expression of *kin10* and *kin11* by virus-induced gene silencing leads to deformed leaves, inflorescences and flowers, short petioles, and reduced activation of stress and starvation genes, while single mutants of *kin10* and *kin11* resemble wild-type plants [42], indicating that SnRK1 functions in development and stress responses.

SnRK1 functions as an energy sensor, using carbohydrates as indicators of plant energy status [32]. For example, high concentrations of sugar phosphates, including trehalose-6-phosphate (T6P), can indicate energy availability and inhibit SnRK1 activity to maintain energy homeostasis [45-47]. The SnRK1 complex can activate basic leucine zipper (bZIP) transcription factors from the C and S family, including bZIP2, bZIP11 and bZIP63, in response to starvation [42, 48, 49]. This in turn leads to upregulation of the expression of genes of various catabolic pathways, including autophagy and degradation of cell wall components, starch, amino acids, sucrose, lipids and protein, providing alternative sources of energy and metabolites [42]. This suggests that SnRK1 has a regulatory influence on global plant metabolism, growth and energy balance [42].

In addition to inhibition of SnRK1 by T6P, type 2C protein phosphatases (PP2C) can also negatively regulate SnRK1 by dephosphorylation [50], leading to its inactivation. Abscisic acid (ABA) inhibits PP2C and therefore can positively regulate SnRK1 by allowing its activation [50]. In contrast with AMPK, SnRK1 has not been shown to be allosterically regulated by AMP, but AMP instead affects its rate of dephosphorylation and therefore activity [38, 51].

Recent studies have shown that Arabidopsis KIN10 interacts with the TOR complex subunit RAPTOR *in vivo* and can phosphorylate RAPTOR *in vitro*, as for its mammalian orthologs [52]. This suggests a crosstalk between these two regulators of energy metabolism and growth. Since the yeast and mammalian orthologs of SnRK1 are positive regulators of autophagy [37], and AMPK can act either through the TOR signaling pathway or independently of it, we hypothesized that KIN10 functions in the regulation of autophagy in

plants, potentially through TOR. Our results demonstrate that KIN10 is an activator of autophagy in *Arabidopsis* and that it acts upstream of TOR in regulation of autophagy.

2.3 Results

2.3.1 Overexpression of KIN10 leads to increased autophagy

AMPK and Snf1, the mammalian and yeast orthologs of SnRK1, have been demonstrated to be positive regulators of autophagy [35-37]. To determine whether SnRK1 can regulate autophagy in *Arabidopsis*, autophagy activity was assessed in the previously described *KIN10* overexpression lines OX-1 and OX-2 [42]. Seven-day-old WT, OX-1 and OX-2 seedlings grown under standard growth conditions were stained with the acidotropic dye monodansycadaverine (MDC) [53] and visualized by confocal microscopy (Fig 1A). We have previously shown that under our conditions, MDC labeling in roots co-localizes with labeling by the specific autophagosome marker GFP-ATG8e, indicating that the fluorescent structures correspond to autophagosomes [54]. While WT seedlings had very few visible autophagosomes, autophagosomes were abundant in both overexpression lines. Autophagy was quantified by counting autophagosomes in epifluorescence images of equal area of the elongation zone of the roots for each genotype (Fig 1B). While WT plants had the expected low basal autophagy activity, the KIN10 overexpression lines had significantly increased autophagy activity when compared to WT. There was no significant difference in autophagy activity between OX-1 and OX-2 (Fig 1B).

To confirm these results, protoplasts were prepared from leaves of 4-6 week-old WT, OX-1 and OX-2 *Arabidopsis* plants, followed by transient expression of GFP-ATG8e to label autophagosomes [53]. The protoplasts were imaged by confocal (Fig S1A) and fluorescence

microscopy, and the percentage of protoplasts with active autophagy was determined (Fig 1C). A protoplast was considered to have activated autophagy if it contained 3 or more GFP-labeled autophagosomes or autophagic bodies [55]. Consistent with the results from MDC staining, a low percentage of protoplasts (12%) from WT plants had active autophagy, while the percentage of protoplasts from KIN10 overexpression lines with active autophagy was significantly higher (30%). There was no difference in the percentage of protoplasts with autophagy between KIN10 overexpression lines (Fig 1C). All genotypes expressed similar levels of GFP-ATG8e (Fig S1B).

As an alternative approach to increasing SnRK1 activity, seedlings were incubated with 5-aminoimidazole-4-carboxamide ribonucleoside monophosphate (AICAR), a chemical activator of AMPK-like kinases [56]. GFP-ATG8e-expressing [14] seedlings were grown under standard conditions on half-strength MS for 7 days and then transferred to the same medium supplemented with 10 mM AICAR for 1 hour. In the presence of AICAR, autophagy activity was significantly increased when compared to the control (Fig 1D). Together, these results suggest that KIN10 is a positive regulator of autophagy in *Arabidopsis* and that an increase in KIN10 activity either by overexpression or by chemical activation leads to increased basal autophagy.

2.3.2 Autophagy is blocked during abiotic stress in a *kin10* mutant

Since overexpression of KIN10 caused constitutive activation of autophagy, we hypothesized that loss of function of KIN10 would have a negative effect on the activation of autophagy. To test this hypothesis, a previously described knockout mutant with a T-DNA insertion in the eleventh exon of the *KIN10* gene [57, 58] was obtained from the Arabidopsis Biological Resource Center. WT and *kin10* mutant seedlings were grown on half-strength

MS plates for seven days, followed by incubation under abiotic stress conditions (salt, osmotic, oxidative, endoplasmic reticulum (ER) stresses or fixed-carbon or nitrogen deficiency) to activate autophagy. Autophagy was detected by MDC staining and quantified by counting the number of autophagosomes per unit area. Under normal conditions, the *kin10* mutant had a low basal level of autophagy, similar to WT seedlings (Fig 2). As expected, autophagy was induced in WT seedlings under salt (Fig 2A), osmotic (Fig 2B), starvation (Fig 2C, 2D), oxidative (Fig 2E), and ER stress (Fig 2 F-G). In the *kin10* mutant, activation of autophagy by stress was completely blocked (Fig 2A, 2 C-F), with the exception of osmotic stress. Upon osmotic stress treatment, activation of autophagy could be observed in the *kin10* mutant, although the degree of activation was significantly reduced when compared to WT (Fig 2B). Confocal images of MDC-stained WT and *kin10* seedlings in control and ER stress conditions are shown as an example (Fig 2G).

To confirm that the loss of *kin10* prevents activation of autophagy under most abiotic stress conditions, protoplasts were prepared from leaves of 4 – 6 week old WT and *kin10* *Arabidopsis* plants, followed by transient expression of GFP-ATG8e to label autophagosomes. Protoplasts were incubated under abiotic stress conditions, followed by quantification of the percentage of protoplasts with active autophagy (Fig 3A-E, Fig S2). Consistent with the MDC staining, activation of autophagy by abiotic stress was observed in protoplasts from WT plants, while those from the *kin10* mutant lacked induction of autophagy under salt (Fig 3A), starvation (Fig 3C), ER (Fig 3D) and oxidative stress (Fig 3E). After osmotic stress, autophagy was induced in both WT and *kin10* mutant protoplasts, although in the *kin10* mutant plants the level of autophagy activity was significantly lower than in WT plants (Fig 3B). Confocal images of WT and *kin10* protoplasts in control and ER

stress conditions are shown as an example (Fig S2A), and both genotypes expressed GFP-ATG8e to similar levels (Fig S2B).

Finally, we confirmed that the autophagy defects observed in the *kin10* mutant were caused by loss of function of the *KIN10* gene. N-terminally FLAG-tagged KIN10 was co-expressed with GFP-ATG8e in leaf protoplasts from *kin10* plants and subjected to ER stress as a representative stress condition. Confocal images of WT, *kin10* and *kin10:*FLAG-KIN10 protoplasts in control and ER stress conditions are shown in Fig S3A, and all genotypes expressed GFP-ATG8e to similar levels (Fig S3B). Unlike the *kin10* mutant expressing GFP-ATG8e alone, autophagy was activated in the FLAG-KIN10-expressing *kin10* protoplasts, indicating that the *KIN10* transgene was able to complement the autophagy phenotype of the *kin10* mutant (Fig 3F, S3A). The degree of autophagy activation in the complemented protoplasts was still significantly lower than that of WT protoplasts, possibly due to differences in expression level. Our results indicate that KIN10 is required for activation of autophagy by abiotic stress, but not for the basal autophagy observed under control conditions.

2.3.3 Inhibition of the SnRK1 complex blocks activation of autophagy by abiotic stress

Trehalose-6-phosphate (T6P) is a sugar synthesized from UDP-glucose and glucose-6-phosphate by trehalose-6-phosphate synthase [59] and has been shown to inhibit the activity of the SnRK1 complex [45]. To confirm that loss of KIN10 activity prevents activation of autophagy during stress, 7-day-old GFP-ATG8e-expressing seedlings were subjected to abiotic stress treatments as described above and co-treated with 0.1 mM T6P for the last 3 hours of the stress treatment (Fig 4). Upon exposure to salt, starvation, oxidative or ER stresses, treatment with T6P prevented activation of autophagy (Fig 4A, 4C-4F).

Consistent with the *kin10* mutant phenotype, treatment with T6P did not completely inhibit the induction of autophagy during osmotic stress, although autophagy activity was significantly reduced when compared to the untreated control (Fig 4B). As examples, confocal images upon ER and salt stress are shown (Fig 4G). These results demonstrate that the activity of the SnRK1 complex is necessary for the induction of autophagy in response to abiotic stress, and SnRK1 is therefore a positive regulator of autophagy.

2.3.4 KIN10 acts upstream of TOR in regulation of autophagy

The mammalian ortholog of KIN10, AMPK, can regulate autophagy through the mTOR signaling pathway or through an mTOR-independent pathway [35, 36]. In plants, TOR has been identified as a negative regulator of autophagy [17], and the TOR complex subunit RAPTOR can be phosphorylated by SnRK1 [52], but the relationship between these components in regulation of autophagy is unknown. We hypothesized that KIN10 acts upstream of TOR in the regulation of autophagy. If our hypothesis is true, then (a) blocking both TOR activity and KIN10 activity will lead to constitutive autophagy and (b) activating both TOR and KIN10 will result in a block in autophagy. We tested this hypothesis using genetic and chemical approaches to inhibit or activate TOR and KIN10.

2.3.4.1 Disruption of TOR and KIN10 activity leads to constitutive autophagy

Previous studies have shown that the chemical AZD8055 (AZD) inhibits TOR kinase activity in *Arabidopsi*s [60] and activates autophagy [26]. Decreased TOR activity leads to constitutive autophagy [17], while a *kin10* mutant is unable to activate autophagy upon abiotic stress (Fig 2). Seven-day-old WT and *kin10* seedlings were transferred to liquid medium supplemented with 10 μM AZD for 3 hours [60], followed by MDC staining. After

AZD treatment, autophagy was active in WT seedlings, consistent with the role of TOR as a negative regulator of autophagy [26]. Upon inhibition of TOR, autophagy was still activated in *kin10* mutant seedlings (Fig 5 A-B).

The TOR complex is composed of the TOR kinase catalytic subunit, RAPTOR, and LST8, in which RAPTOR delivers the substrate to the TOR kinase. In *Arabidopsis,* two *RAPTOR* genes have been identified, *RAPTOR1A* and *RAPTOR1B* [19]*,* and disruption of *RAPTOR1B* results in the inactivation of the TOR complex [26]. Seven-day-old WT and *raptor1b* seedlings were transferred to liquid medium supplemented with 0.1 mM T6P for 3 hours, followed by MDC staining. Under control conditions *raptor1b* seedlings have constitutive autophagy when compared to WT, consistent with disruption of TOR activity. Upon inhibition of SnRK1 by T6P, autophagy activity in the *raptor1b* mutant was not affected (Fig 5 C-D).

To confirm these genetic results using chemical inhibition of TOR and KIN10, 7-day-old GFP-ATG8e seedlings were transferred to liquid medium and treated with AZD, T6P or co-treated with AZD and T6P for 3 hours. Under control conditions autophagy was at a low basal level. Upon inhibition of TOR by AZD, autophagy was activated. Autophagy activity was not affected in seedlings treated with T6P. Upon co-treatment with AZD and T6P, autophagy activity was induced (Fig 5 E-F). These data are all consistent with our hypothesis that KIN10 is upstream of TOR in the regulation of autophagy.

2.3.4.2 Activation of both TOR and KIN10 blocks autophagy

As a second approach to test our hypothesis that KIN10 is upstream of TOR for activating autophagy, the effect of activation of SnRK1 on seedlings with increased TOR activity was assessed. Seven-day-old WT and TOR overexpressing (OE TOR) [61] seedlings

were transferred to liquid medium supplemented with 10 mM AICAR (SnRK1 activator) for 1 hour, followed by MDC staining. Under control conditions the autophagy activity in WT and OE TOR seedlings was at a low basal level. After AICAR treatment, WT seedlings have induced autophagy activity. Upon activation of SnRK1 by AICAR, seedlings overexpressing TOR are unable to activate autophagy (Fig 5G).

Recent studies have shown that the phytohormone auxin can activate TOR kinase [62] and therefore inhibit autophagy [26]. Seven-day-old WT and KIN10 OX-1 and OX-2 seedlings were transferred to liquid medium supplemented with 20 nM NAA for 6 hours, followed by MDC staining. Under control conditions the autophagy activity in WT seedlings was at a low basal level, while KIN10 overexpression seedlings have constitutive autophagy. After NAA treatment, the autophagy activity in KIN10 overexpression lines was significantly reduced (Fig 5H).

Finally, 7-day-old GFP-ATG8e seedlings were transferred to liquid medium and treated with NAA for 6 hours, AICAR for 1 hour, or co-treated with NAA for 6 hours plus AICAR for the last hour of incubation. Under control conditions or upon treatment with NAA, autophagy was at a low basal level. Upon activation of SnRK1 by AICAR, autophagy was activated. Upon co-treatment with AICAR and NAA, induction of autophagy by AICAR was blocked (Fig 5I).

In summary, activation of autophagy by increasing KIN10 activity is blocked upon activation of TOR, whereas disruption of KIN10 activity does not block the constitutive autophagy seen upon inhibition of TOR. Taken together, these results demonstrate that KIN10 acts upstream of TOR in the regulation of autophagy.

2.4 Discussion

Autophagy is a vacuolar degradation pathway induced by multiple environmental stresses in plants, including nutrient starvation, osmotic, oxidative and ER stress, and during certain stages of development such as senescence [1]. Regulation of autophagy has been widely studied in animals and yeast, but is still poorly understood in plants. A few regulators of autophagy have been identified in plants, such as the ATG1/ATG13 complex, which activates autophagy in response to nutrient stress [25], IRE1b, which functions in response to ER stress [12], PTEN, which regulates autophagy in pollen tubes [63], and the TOR complex, a negative regulator of autophagy under nutrient-rich conditions [17]. In this paper, we demonstrate that the SnRK1 complex catalytic subunit KIN10 is a positive regulator of autophagy and that it functions upstream of the TOR complex in the activation of autophagy.

The SnRK1 complex acts as an energy sensor and is activated under conditions of low energy or metabolic stress to inhibit growth and conserve energy [64]. SnRK1 regulates metabolism by the phosphorylation and inactivation of important plant metabolic enzymes, including 3-hydroxymethyl-3-methylglutaryl-CoA reductase [65, 66], sucrose phosphate synthase, nitrate reductase [67] and trehalose phosphate synthase 5 [68]. SnRK1 also indirectly controls carbohydrate metabolism by modulating the transcription of genes such as sucrose synthase, involved in sucrose degradation, and α-amylase, involved in starch degradation [69]. In response to low energy conditions, SnRK1 mammalian and yeast orthologs can activate autophagy via inactivation of the TOR complex [37]. We found that the overexpression of the *KIN10* gene results in constitutive activation of the autophagy pathway in *Arabidopsis*. Furthermore, addition of the AMPK activator AICAR [56] to

seedlings to activate the SnRK1 complex led to induction of autophagy. The activation of SnRK1 results in the upregulation of catabolism and downregulation of anabolism [59], and autophagy is potentially one of the mechanisms used to maintain energy balance. In low energy conditions, autophagy can recycle cytoplasmic components, producing both raw materials that can be used in biosynthetic pathways when substrates are limiting, and alternative TCA cycle substrates for ATP production, thus helping to maintain homeostasis.

Analysis of a *kin10* knockout mutant revealed that KIN10 is necessary to activate autophagy in response to nutrient starvation, salt stress, oxidative stress and ER stress. We hypothesize that upon nutrient deprivation SnRK1 is activated by low energy and in turn activates autophagy to compensate for the nutrient deficiency, thus contributing to stress tolerance. SnRK1 has also been linked to other plant stress responses, including salt tolerance [70] and pathogen resistance [71, 72]. During salt stress, abscisic acid (ABA) levels are increased [73], which potentially can lead to the activation of the SnRK1 complex [50], therefore promoting autophagy. ABA signaling is also critical for osmotic stress responses, which are activated either through ABA-dependent or ABA-independent but DREB2-dependent pathways [74]. During osmotic stress, activation of autophagy in the *kin10* mutant was only partially blocked, indicating that an alternative pathway may exist for activating autophagy under these conditions. One possibility we considered is that the second isoform of the SnRK1 catalytic subunit, KIN11 [42], can substitute for KIN10 under some conditions, including osmotic stress. Previous work has shown that the two isoforms can act antagonistically [44], suggesting that each protein has specific functions. However, a *kin10 kin11* double mutant results in lethality [42], suggesting some degree of functional overlap.

T6P inhibits SnRK1 complex activity in *Arabidopsis*, including both KIN10- and KIN11- containing complexes [45]. T6P reduces SnRK1 activity by ~80%, and even at higher concentrations (up to 4 mM) does not completely block activity [45]. Incubation of *Arabidopsis* seedlings with T6P inhibited autophagy activation in response to nutrient starvation, salt stress, oxidative stress and ER stress, consistent with the *kin10* mutant phenotype. Inhibition of SnRK1 by T6P resulted in only partial inhibition of autophagy activity in response to osmotic stress, as also seen in the *kin10* mutant. The presence of KIN11 therefore cannot account for the partial activation of autophagy observed under osmotic stress in the *kin10* mutant. We hypothesize that autophagy can be regulated by two parallel pathways during osmotic stress: one that is SnRK1-dependent and another that is SnRK1-independent.

TOR negatively regulates autophagy in many organisms, including *Arabidopsis* [17], and regulation of autophagy by AMPK can occur either through TOR or independently of TOR [36, 75]. AMPK regulates autophagy during nutrient starvation by phosphorylating RAPTOR, resulting in the inhibition of TOR and therefore activating autophagy [75]. Recent studies have shown that KIN10 can interact with RAPTOR1B *in vivo* and phosphorylate it *in vitro* [52]. We show here that activation of autophagy by increasing KIN10 activity is blocked upon activation of TOR, whereas disruption of KIN10 activity does not block the constitutive autophagy seen upon inhibition of TOR. Our results demonstrate that KIN10 acts upstream of TOR in the regulation of autophagy in *Arabidopsis* via a TOR-dependent pathway.

The activation of autophagy by ER or oxidative stress is not blocked by activation of TOR, suggesting that it occurs via a TOR-independent pathway [26]. Under ER stress

conditions, autophagy is activated by the accumulation of unfolded proteins [55] and requires the unconventional splicing factor IRE1, although it is independent of IRE1's downstream splicing target bZIP60 [12]. The signaling pathway for activation of autophagy during ER stress therefore appears to be distinct from that during nutrient stress, although it still requires SnRK1 activity. This suggests that, like AMPK and Snf1, KIN10 can also regulate autophagy through a TOR-independent pathway under specific conditions.

In conclusion, we have identified KIN10 as a positive regulator of autophagy in *Arabidopsis*. KIN10 is necessary for the activation of autophagy by many abiotic stresses, either through TOR-dependent or TOR-independent pathways, depending on the stress. In the TOR-dependent pathway, KIN10 acts upstream of TOR, probably by phosphorylating the TOR complex, to positively regulate autophagy. Further work is necessary to determine the exact role of the SnRK1 complex in the regulation of autophagy in response to osmotic stress, to clarify the mechanism by which KIN10 can regulate autophagy independently of TOR, and to identify downstream targets of TOR that contributes to the regulation of autophagy in plants.

2.5 Materials and Methods

2.5.1 Plant materials and growth conditions

Wild-type (Col-0), GFP-ATG8e [14], *kin10* (SALK_127939C*)* [57, 58], KIN10 OX-1, KIN10 OX-2 [42], TOR overexpression [61] and *raptor1b* (SALK_078159) [18] *Arabidopsis thaliana* seeds were surface sterilized with 0.1% (v/v) Triton X-100, 33% (v/v) bleach solution for 20 minutes, rinsed 5 times with sterile water, and kept at 4°C in the dark for at least 2 days. Plants were grown on half-strength MS solid medium (Murashige-Skoog

with vitamins mixture [Caisson, MSP09], 1% sucrose, 2.4 mM MES [pH 5.7], 0.6% Phytoblend agar) or in soil under long day conditions (16 h light) at 22°C.

2.5.2 Stress and chemical treatments

For salt stress, 7-day-old seedlings were transferred to half-strength MS liquid medium with 160 mM NaCl for 6 hours [11]. For osmotic stress, 7-day-old seedlings were transferred to half-strength MS liquid medium with 350 mM mannitol for 6 hours [11]. For oxidative stress, 7-day-old seedlings were transferred to half-strength MS liquid medium supplemented with 10 mM hydrogen peroxide for 2 hours [14]. For ER stress treatment, 7-day-old seedlings were transferred to half-strength MS liquid medium supplemented with 2 mM dithiothreitol (DTT) for 6 hours [12]. For starvation stress, 7-day-old seedlings were transferred to half-strength MS plates lacking sucrose or nitrogen for 4 days. Plants grown on sucrose starvation plates were incubated in the dark.

For SnRK1 inhibitor treatment, 7-day old seedlings were transferred to half-strength MS liquid medium supplemented with 0.1 mM trehalose-6-phosphate (T6P) [Santa Cruz, SC216004] for 3 hours [45]. For treatment in the presence of stress, seedlings were subjected to the abiotic stress conditions as described above and T6P was added to a final concentration of 0.1 mM for the last 3 hours of the treatment. Seedlings under starvation stress were transferred after the 4 days of starvation to half-strength MS liquid medium lacking sucrose or nitrogen and supplemented with 0.1 mM T6P for 3 hours.

For SnRK1 activation, 7-day-old seedlings were transferred to half-strength MS liquid medium supplemented with 10 mM 5-aminoimidazole-4-carboxamide ribonucleoside monophosphate (AICAR) [EMD Millipore Calbiochem, 12304125MG] for 1 hour [56].

For TOR inhibitor treatment, 7-day-old seedlings were transferred to half-strength MS liquid medium supplemented with 10 μM AZD8055 (AZD) or DMSO as solvent control for 3 hours [60].

For TOR activation treatment, 7-day-old seedlings were transferred to half-strength MS liquid medium supplemented with 20 nM 1-naphthaleneacetic acid (NAA) [Sigma, N1641] or DMSO as solvent control for 6 hours.

2.5.3 MDC staining and microscopy

Arabidopsis seedlings were stained with 0.05 mM monodansylcadaverine (MDC) [Sigma, 30432] for 10 minutes, followed by 3 brief washes with phosphate-buffered saline [53]. Seedlings were observed using a Zeiss Axioplan II compound microscope equipped with an Axio Cam HRC digital imaging system at the Iowa State University Microscopy and Nanoimaging Facility, using a X40 objective and a 4’, 6-diamidino-2-phenylindole (DAPI) filter. GFP fluorescence was imaged using a Zeiss AxioImager microscope with a X40 objective and a fluorescein isothiocyanate (FITC) filter at the Iowa State Microscopy and Nanoimaging Facility.

Confocal microscopy was performed using a Leica SP5 confocal laser scanning microscope with a X63 oil immersion objective at the Iowa State University Confocal and Multiphoton Facility. The excitation and emission wavelengths for GFP were 488 and 507 nm respectively. The excitation and emission wavelengths for MDC were 435 and 455 nm respectively.

2.5.4 Transient transformation of leaf protoplasts

Leaf protoplasts were prepared from 4 – 6- week- old *Arabidopsis* plants [76], and 20 μg of plasmid DNA was used for each transformation. After transformation, protoplasts were incubated at room temperature overnight in darkness to allow expression, with 40 rpm orbital shaking, followed by stress and/or addition of chemicals to the protoplast suspension. Concentrations and incubation times were as described above (stress and chemical treatments section). Autophagosomes labeled with GFP-ATG8e were counted under an epifluorescence microscope. A protoplast was considered to have active autophagy if 3 or more GFP-ATG8e-labeled autophagosomes were detected [55].

For immunoblotting, protoplasts were collected by centrifugation at 300 rpm. Protein was dissolved in 3X SDS loading buffer [6% (w/v) SDS, 20% (v/v) glycerol, 125 mM Tris-HCl pH 6.8]. Proteins were separated by 12% SDS-polyacrylamide gel electrophoresis and analyzed by western blot using anti-GFP antibody [Life Technologies, A11122].

2.5.5 Image analysis

Autophagosomes labeled with MDC or GFP-ATG8e in the elongation zone in the roots were manually counted, and only individual motile dots were counted as autophagosomes. GFP-ATG8e puncta in leaf protoplasts were directly counted under fluorescence microscopy.

2.5.6 Generation of FLAG-KIN10

The *KIN10* cDNA was synthesized by RT-PCR from total RNA from 10-day-old seedlings grown on half strength MS plates, using gene-specific primers (forward 5’-CACCGGTACCGATTACAAGGATGACGACGATAAGATGGATGGATCAGGCACAGG

-3’, reverse 5’-AACACCGAGCTCTCAGAGGACTCGGAGCTGAG-3’). The forward primer also encoded the FLAG tag for detection of expression. The cDNA was ligated into the MCS11 binary vector using KpnI and SacI restriction sites. The final construct was verified by enzymatic digestion and sequencing.

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2.8 Figures

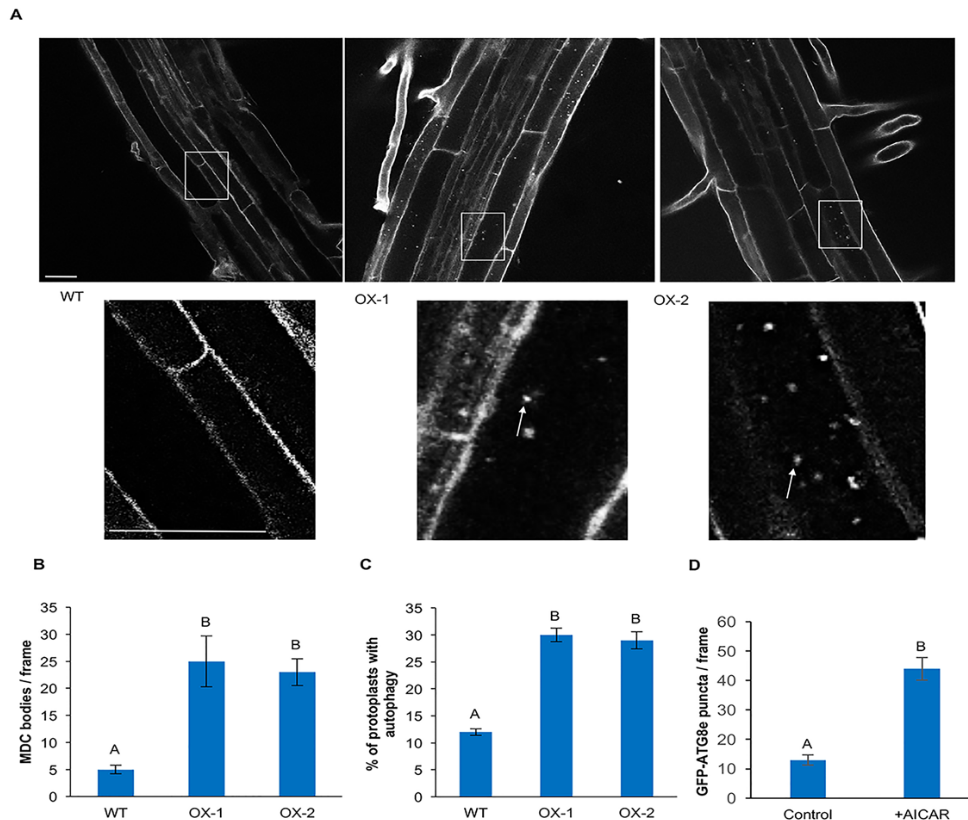


Figure 1. Overexpression of KIN10 leads to increased basal autophagy

(A) WT, OX-1 and OX-2 seedlings were grown on standard growth medium for 7 days, then stained with MDC. Confocal microscopy was used to visualize autophagosomes in roots. The insets show enlargements of the indicated boxes. Arrows indicate MDC-labeled structures. Scale bars = 20 µm. **(B)** Quantification of the number of autophagosomes in seedlings grown as in (A). KIN10 overexpression lines have increased autophagy activity when compared to WT. Different letters denote statistical significance for three biological replicates with at least 10 images per replicate, $p<0.05$, t-test. Error bars indicate standard error. **(C)** The autophagosome marker GFP-ATG8e was transiently expressed in leaf protoplasts from the indicated genotypes and the percentage of protoplasts with active autophagy determined. A protoplast was considered to have active autophagy if it contained 3 or more GFP-ATG8e-labeled autophagosomes. KIN10 overexpression lines have increased autophagy activity when compared to WT. Different letters denote statistical significance for three biological replicates, with 100 protoplasts per sample per replicate, $p<0.05$, t-test. Error bars indicate standard error. **(D)** Seven-day-old GFP-ATG8e-expressing seedlings were transferred to liquid medium plus or minus 10 mM AICAR for 1 hour, and the number of autophagosomes per unit area counted. Seedlings treated with AICAR had higher autophagy activity than the control. Different letters denote statistical significance for three biological replicates with at least 10 images per replicate, $p<0.05$, t-test. Error bars indicate standard error.

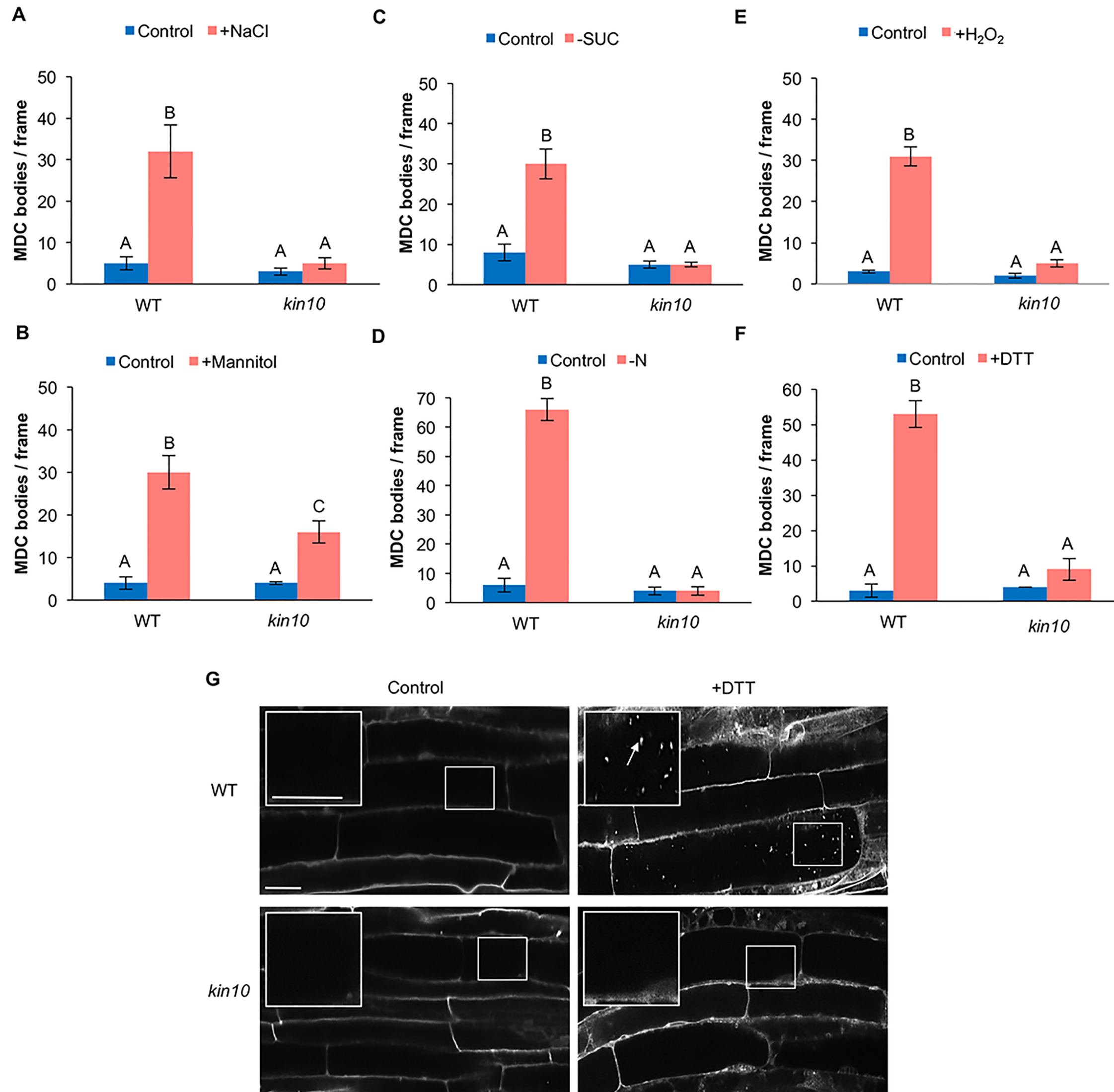


Figure 2. Autophagy is blocked during abiotic stress in *kin10* mutant seedlings

Seven-day-old WT and *kin10* seedlings were transferred to ½ MS liquid medium supplemented with 160 mM NaCl for 6 hours **(A)**, ½ MS liquid medium supplemented with 350 mM mannitol for 6 hours **(B)**, ½ MS plates lacking sucrose for 4 days in the dark **(C)**, ½ MS plates lacking nitrogen for 4 days **(D)**, ½ MS liquid medium supplemented with 10 mM hydrogen peroxide for 2 hours **(E)**, or ½ MS liquid medium supplemented with 2 mM DTT (ER stress) for 6 hours **(F)**. Seedlings were stained with MDC and autophagosomes counted. Autophagy was activated in WT seedlings after abiotic stress, while in *kin10* mutant seedlings autophagy was not induced in most conditions. The exception was osmotic stress, in which activation of autophagy in the *kin10* mutant was reduced but not completely blocked. Different letters denote statistical significance, $p<0.05$, t-test. Error bars indicate standard error. **(G)** Confocal images of WT and *kin10* mutant roots under control conditions and ER stress as a representative stress. The insets show enlargements of the indicated boxes. White arrows point to autophagosomes. Scale bars = 20 µm.

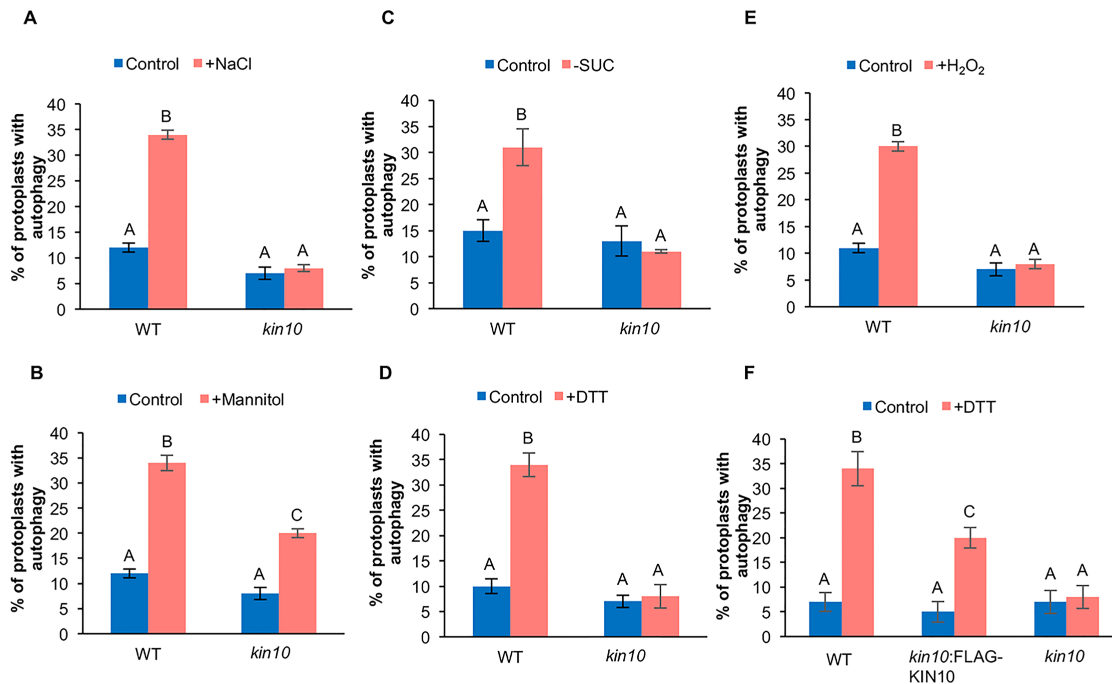


Figure 3. Autophagy is blocked during abiotic stress in *kin10* mutant protoplasts

WT and *kin10* protoplasts were transiently transformed with the autophagy marker GFP-ATG8e, incubated overnight to allow expression, and then the protoplast solution was supplemented with 160 mM NaCl for 6 hours **(A)**, supplemented with 350 mM mannitol for 6 hours **(B)**, incubated plus or minus 1% sucrose for 48 hours **(C)**, supplemented with 2 mM DTT for 6 hours **(D)**, or supplemented with 10 mM hydrogen peroxide for 2 hours **(E)**. Autophagosomes were visualized by epifluorescence microscopy and the percentage of protoplasts with active autophagy determined. Different letters denote statistical significance for three biological replicates with 100 protoplasts for each sample per replicate, $p<0.05$, t-test. Error bars indicate standard error. Autophagy was activated in WT protoplasts after abiotic stress, but not in *kin10* mutant protoplasts. Upon osmotic stress, activation of autophagy in the *kin10* mutant was reduced but not completely blocked. **(F)** Protoplasts were co-transformed with FLAG-KIN10 and GFP-ATG8e constructs to confirm that the lack of autophagy in *kin10* was due to disruption of the *KIN10* gene. DTT was used to induce autophagy as in (D). Expression of FLAG-KIN10 restored the induction of autophagy during ER stress in the *kin10* mutant. Different letters denote statistical significance for three biological replicates with 100 protoplasts for each sample per replicate, $p<0.05$, t-test. Error bars indicate standard error.

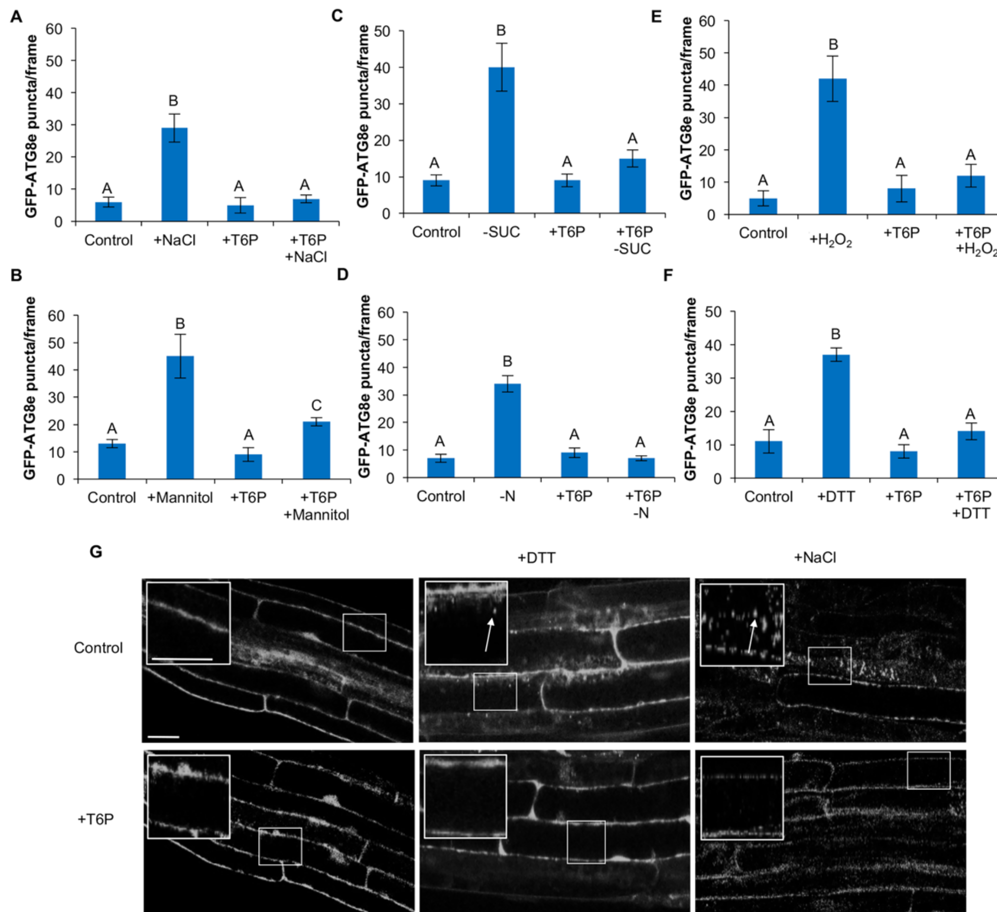


Figure 4. Inhibition of SnRK1 activity by T6P inhibits autophagy under abiotic stress

Seven-day-old GFP-ATG8e seedlings were transferred to ½ MS liquid medium supplemented with 0.1 mM T6P for 3 hours as control, or liquid medium supplemented with 160 mM NaCl for 6 hours and 0.1 mM T6P for the last 3 hours of treatment **(A)**, liquid medium supplemented with 350 mM mannitol for 6 hours and 0.1 mM T6P for the last 3 hours of treatment **(B)**, ½ MS plates lacking sucrose for 4 days in the dark followed by 0.1 mM T6P treatment in liquid medium for 3 hours **(C)**, ½ MS plates lacking nitrogen for 4 days followed by 0.1 mM T6P treatment in liquid medium for 3 hours **(D)**, liquid medium supplemented with 0.1 mM T6P for 3 hours and 10 mM hydrogen peroxide added for the last 2 hours **(E)**, or liquid medium supplemented with 2 mM DTT for 6 hours and 0.1 mM T6P for the last 3 hours of treatment **(F)**. Autophagosomes were imaged using epifluorescence microscopy and counted. Addition of T6P blocked the activation of autophagy in most conditions. In osmotic stress, autophagy was reduced but not completely blocked by T6P. Different letters denote statistical significance for three biological replicates with at least 10 frames per replicate, $p<0.05$, t-test. Error bars indicate standard error. **(G)** Confocal images of roots of GFP-ATG8e-expressing seedlings under control conditions, ER stress and salt stress as representative stresses. The insets show enlargements of the indicated boxes. White arrows point to autophagosomes. Scale bars = 20 μm.

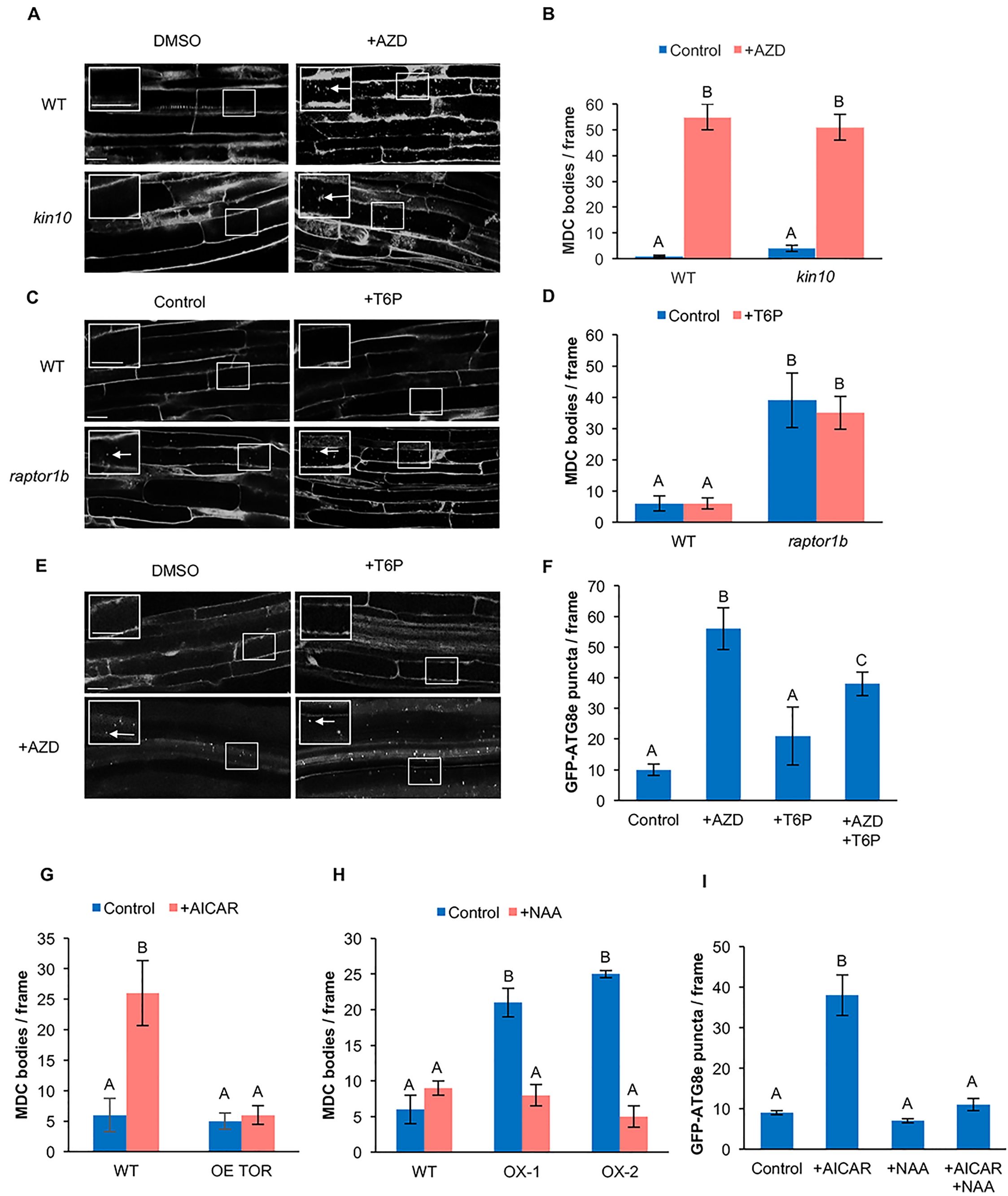
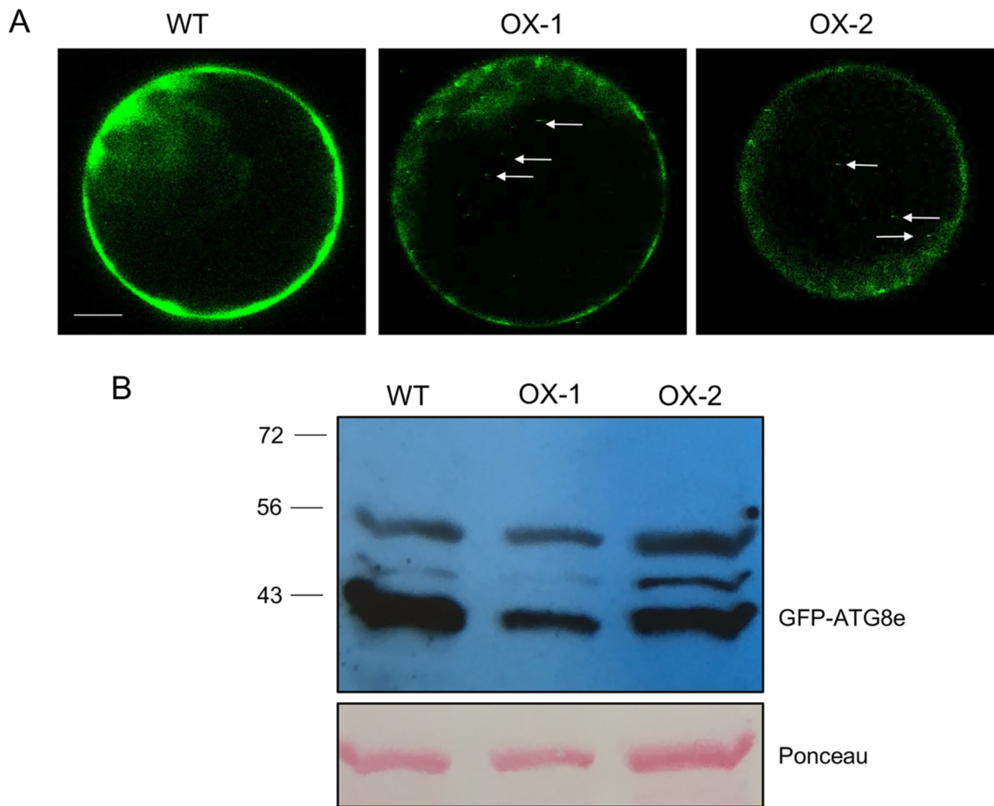


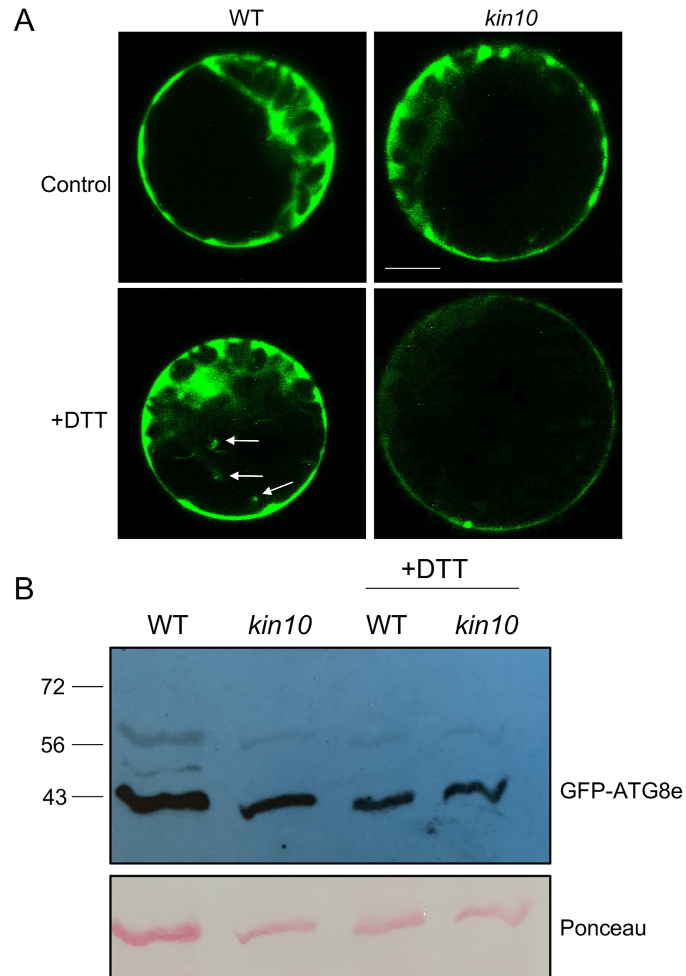
Figure 5. SnRK1 acts upstream of TOR in the autophagy pathway

(A) WT and *kin10* seedlings were grown on ½ MS plates for 7 days. Seedlings were transferred to ½ MS liquid medium supplemented with 10 μM AZD or DMSO for 3 hours, followed by MDC staining. Confocal microscopy was used to visualize autophagosomes (white arrows) in roots. The insets show enlargements of the indicated boxes. Scale bars = 20 μm. **(B)** Quantification of autophagy activity as shown in (A). Upon inhibition of TOR with AZD, autophagy was still activated in *kin10* mutant seedlings. **(C)** WT and *raptor1b* seedlings were grown on ½ MS plates for 7 days. Seedlings were transferred to ½ MS liquid medium supplemented with 0.1 mM T6P for 3 hours, followed by MDC staining. Confocal microscopy was used to visualize autophagosomes (white arrows) in roots. The insets show enlargements of the indicated boxes. Scale bars = 20 μm. **(D)** Quantification of autophagy activity in (C). Upon inhibition of SnRK1 with T6P, autophagy activity was not affected in *raptor1b* seedlings. **(E)** GFP-ATG8e seedlings were grown on ½ MS plates for 7 days. Seedlings were transferred to ½ MS liquid medium supplemented with 0.1 mM T6P or 10 μM AZD or T6P plus AZD for 3 hours. Confocal microscopy was used to visualize autophagosomes (white arrows) in roots. The insets show enlargements of the indicated boxes. Scale bars = 20 μm **(F)** Quantification of autophagosomes labeled with GFP-ATG8e in (E). Upon inhibition of both TOR and SnRK1, autophagy was activated. **(G)** WT and OE TOR seedlings were grown on ½ MS plates for 7 days. Seedlings were transferred to ½ MS liquid medium supplemented with 10 mM AICAR for 1 hour, followed by MDC staining, and autophagosomes counted. Overexpression of TOR was able to suppress AICAR-induced autophagy. **(H)** WT, KIN10 OX-1 and KIN10 OX-2 seedlings were grown on ½ MS plates for 7 days. Seedlings were transferred to ½ MS liquid medium supplemented with 20 mM NAA or DMSO for 6 hours, stained with MDC and autophagosomes counted. Activation of TOR by auxin inhibited the constitutive autophagy in KIN10 overexpression lines. **(I)** Seven-day-old GFP-ATG8e seedlings were transferred to ½ MS liquid medium supplemented with 10 mM AICAR or 20 nM NAA or both AICAR and NAA. Activation of TOR by NAA blocked induction of autophagy by AICAR. For all graphs, different letters denote statistical significance for three biological replicates with at least 10 frames per replicate, $p<0.05$, t-test. Error bars indicate standard error.



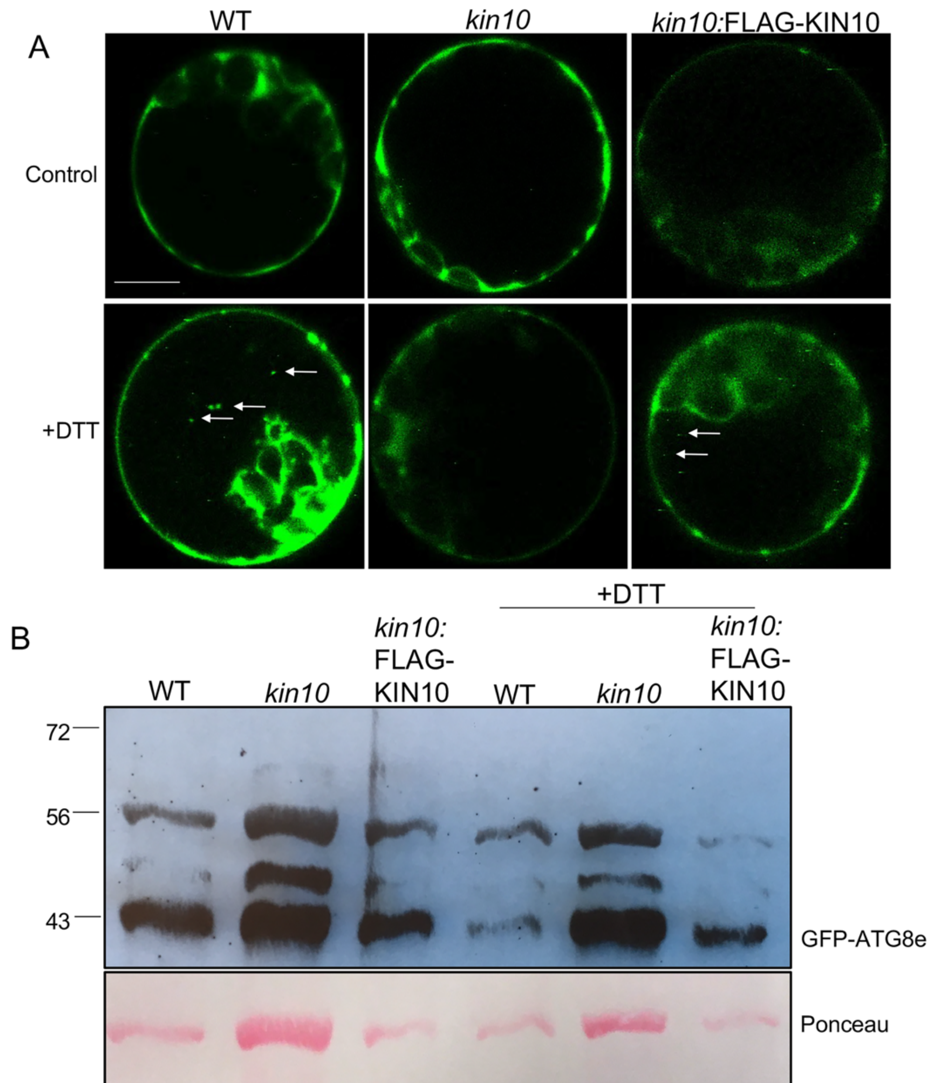
Supplemental Figure 1. Transient expression of GFP-ATG8e in KIN10 overexpression lines

(A) The autophagosome marker GFP-ATG8e was transiently expressed in leaf protoplasts from the indicated genotypes and visualized by confocal microscopy. KIN10 overexpression lines have increased autophagy activity when compared to WT. White arrows point to autophagosomes. Scale bar = 10 μm. **(B)** Immunoblotting of protein extracts from protoplasts as in (A) using antibodies against GFP. Ponceau S stain was used as loading control. All samples show approximately equal expression of GFP-ATG8e.



Supplemental Figure 2. Transient expression of GFP-ATG8e in *kin10* mutant protoplasts

(A) The autophagosome marker GFP-ATG8e was transiently expressed in leaf protoplasts from the indicated genotypes and visualized by confocal microscopy. After inducing ER stress as a representative stress with 2 mM DTT, the *kin10* mutant fails to activate autophagy when compared to WT. White arrows point to autophagosomes. Scale bar = 10 µm. **(B)** Immunoblotting of protein extracts from protoplasts as in (A) using antibodies against GFP. Ponceau S stain was used as loading control. All samples show approximately equal expression of GFP-ATG8e.



Supplemental Figure 3. Complementation of the *kin10* mutant

(A) The autophagosome marker GFP-ATG8e was transiently expressed in leaf protoplasts from the indicated genotypes and visualized by confocal microscopy. After inducing ER stress as a representative stress with 2 mM DTT, *kin10*:FLAG-KIN10 shows induction of autophagy as in WT, while *kin10* mutant fails to induce autophagy. White arrows point to autophagosomes. Scale bar = 10 µm. **(B)** Immunoblotting of protein extracts from protoplasts as in (A) using antibodies against GFP. Ponceau S stain was used as loading control. All samples show approximately equal expression of GFP-ATG8e.

CHAPTER 3. GLUTATHIONE IS REQUIRED FOR ACTIVATION OF AUTOPHAGY DURING SALT STRESS AND NUTRIENT STARVATION

3.1 Abstract

Autophagy is a degradation process in which cells break down and recycle their cytoplasmic contents during environmental stress or development. Glutathione (GSH) is an antioxidant that prevents cellular damage in the cell and it has been shown to negatively regulate autophagy in mammals and mitophagy in yeast in response to starvation. We therefore hypothesized that GSH regulates autophagy in response to abiotic stress in *Arabidopsis*. To test this hypothesis, we used the chemical inhibitor of GSH synthesis, buthionine sulfoximine (BSO), and the glutathione-deficient mutant *cad2-1* to analyze autophagy when GSH is decreased in *Arabidopsis*. Autophagy activity was induced after all abiotic stresses. BSO treatment inhibited autophagy induction by salt stress and sucrose starvation, while autophagy activation was not affected during oxidative and osmotic stresses. Addition of exogenous GSH while inhibiting GSH synthesis with BSO under salt stress or sucrose starvation rescued the autophagy activation. A *cad2-1* mutant had a basal level of autophagy under control conditions similar to wild-type plants, but activation of autophagy by salt and starvation stresses was blocked. Addition of exogenous GSH rescued autophagy activation under those conditions. These results demonstrate that GSH is required for autophagy activation under salt stress and nutrient starvation conditions, but not during oxidative stress or osmotic stress. Autophagy during salt and starvation stress is regulated in a NADPH-oxidase dependent manner and TOR, a negative regulator of autophagy, can act either downstream of NADPH oxidase or in an NADPH oxidase-independent pathway. We tested TOR and the SnRK1 complex, a positive regulator of autophagy, as possible

candidates for GSH regulation. Inactivation of TOR kinase by mutation of the *RAPTOR1B* gene and overexpressing the gene of the SnRK1 catalytic subunit, *KIN10*, resulted in autophagy activation. Inhibition of GSH synthesis by BSO did not affect the autophagy activation in *raptor1b* mutants. Inhibition of GSH synthesis by BSO resulted in inhibition of autophagy activity in the KIN10 overexpression seedlings. Addition of exogenous GSH while inhibiting GSH synthesis with BSO to KIN10 overexpression seedlings rescued the autophagy activation. Our results suggest that GSH acts through the SnRK1 complex in the activation of autophagy.

3.2 Introduction

Macroautophagy (hereafter autophagy) is a degradation process within the cell that recycles cytoplasmic contents when cells are under stress or during development. Autophagy is characterized by the formation of a double membrane vesicle, called an autophagosome, around the targeted cargo and delivery of the cargo into the vacuole/lysosome for degradation and recycling (Soto-Burgos et al., 2018). Major proteins functioning in autophagosome formation include the autophagy-related (ATG)1 complex (Kamada et al., 2000), two ubiquitin-like conjugates (ATG12-ATG5 and ATG8-PE) (Yin et al., 2016), and ATG9 (Reggiori et al., 2005). The process of autophagy is conserved among eukaryotes and is highly regulated. Among the major regulators that have been identified are the ATG1 complex itself (Kamada et al., 2000), the target of rapamycin (TOR) complex (Noda and Ohsumi, 1998; Pattingre et al., 2008) and AMP-activated protein kinase (AMPK) (Wang et al., 2001; Egan et al., 2011; Kim et al., 2011). Regulators of autophagy in plants include the plant ortholog of AMPK, the Snf1-related protein kinase 1 (SnRK1) complex, which can

positively regulate autophagy (Chen et al., 2017; Soto-Burgos and Bassham, 2017), the ATG1 complex, a positive regulator of autophagy (Suttangkakul et al., 2011) and the TOR complex, a negative regulator of the autophagy pathway (Liu and Bassham, 2010).

In plants, autophagy has been mostly studied in relation to stress tolerance. Under normal conditions, cells maintain a basal level of autophagy to turn over damaged organelles or proteins (Yang and Bassham, 2015). Autophagy is upregulated during biotic and abiotic stresses including nutrient deficiency (Doelling et al., 2002; Hanaoka et al., 2002), salt and drought stress (Liu et al., 2009), endoplasmic reticulum (ER) stress (Liu et al., 2012), oxidative stress (Xiong et al., 2007), pathogen attack (Liu et al., 2005; Lai et al., 2011; Lenz et al., 2011; Haxim et al., 2017), hypoxia (Chen et al., 2015) and heat stress (Zhou et al., 2013; Yang et al., 2016). Under many of these stresses, the production of reactive oxygen species (ROS) by nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase acts as a signal to activate defense and stress response pathways (Chen and Schopfer, 1999; Torres et al., 2002). It has been shown that autophagy can be regulated through NADPH-oxidase dependent (starvation, salt stress) or independent pathways (ER and osmotic stress), depending on the specific stress conditions (Liu et al., 2009; Liu et al., 2012).

Glutathione (GSH) is an important antioxidant that prevents damage to cellular components caused by ROS (Pompella et al., 2003). There are two forms of glutathione, the oxidized (GSSG) form and the reduced (GSH) form, and the ratio between reduced and oxidized forms of GSH is an indicator of the redox environment (Dickinson and Forman, 2002). Under physiological conditions, reduced GSH is the major form, with its concentration 10 – 100 fold higher than the oxidized species (Hwang et al., 1992). The majority of the GSH is in the cytosol, which is the main site of its synthesis (Lu, 2013).

Biosynthesis of GSH occurs in two successive steps, catalyzed by the enzymes γ-glutamylcysteine synthetase and GSH synthetase, from its constituent amino acids, glutamic acid, cysteine and glycine (Alscher, 1989). Once synthesized, GSH is delivered to some intracellular compartments, including the nucleus, mitochondria and endoplasmic reticulum, and to the extracellular space to be used by other cells and tissues. GSH has many roles in the cell besides scavenging radicals, including storage of cysteine reserves, maintaining the essential thiol status of cysteine residues on proteins, participation in the production of deoxyribonucleotides, and signal transduction (Aquilano et al., 2014).

GSH has been shown to be involved in the regulation of autophagy and selective autophagy of mitochondria (mitophagy) in mammals and yeast. In yeast, decreased levels of GSH increase the rate of mitophagy (Deffieu et al., 2009). The GSH precursor, N-acetylcysteine, was able to inhibit autophagy by impeding the microtubule-associated protein 1 light chain 3 (LC3) (animal ATG8 homolog) delipidation by ATG4 in mammals, which is essential for autophagosome expansion and the normal progression of autophagy (Scherz-Shouval et al., 2007). N-acetylcysteine affected mitophagy by prevented delivery of mitochondria to the vacuoles in yeast (Deffieu et al., 2009). Furthermore, nutrient starvation induces GSH efflux through the cell membrane to the extracellular space, altering the cellular redox environment by thiol unbalance, which induces autophagy (Desideri et al., 2012).

In plants, a connection between autophagy and GSH has not been reported. We hypothesized that GSH, being an antioxidant, will decrease the ROS levels produced during abiotic stress, in turn leading to decreased autophagy. Here, we used the chemical inhibitor of GSH, buthionine sulfoximine (BSO) and the glutathione-deficient mutant *cad2-1* to test our hypothesis. Our results demonstrate that GSH synthesis is required for autophagy activation

under salt stress and nutrient starvation conditions, but not during oxidative stress or osmotic stress. Furthermore, our results suggest that GSH acts through the SnRK1 complex in the activation of autophagy.

3.3 Results

3.3.1 BSO reduces GSH content in *Arabidopsis*

GSH is an important antioxidant that prevents damage to cellular components caused by ROS (Pompella et al., 2003). We hypothesized that GSH will detoxify the ROS produced during abiotic stress, leading to decreased autophagy. To test our hypothesis, we initially chose a chemical approach, using buthionine sulfoximine (BSO) and GSH. BSO inhibits the enzyme γ-glutamylcysteine synthetase, which catalyzes the first step of GSH synthesis (Mendum et al., 1990). To test the effect of BSO on GSH levels in *Arabidopsis,* ten-day-old wild-type seedlings were treated with 1 mM BSO or co-treated with BSO plus 2 mM GSH for 6 hours. Following treatment, GSH was extracted from the seedlings and analyzed by HPLC. BSO treatment reduced the endogenous GSH concentration by 50% when compared to untreated controls (Figure 1). Addition of exogenous GSH along with BSO treatment significantly increased the GSH content, demonstrating that exogenous GSH can be taken up by the seedlings (Figure 1). Together, these results showed that BSO can reduce the endogenous GSH concentration in *Arabidopsis* seedlings and that addition of exogenous GSH in the presence of BSO can restore the GSH content.

3.3.2 GSH is required for autophagy activation during salt stress and nutrient starvation

The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is an enzyme that produces ROS as signaling molecules under various abiotic and biotic stresses (Jiang and Zhang, 2002). Autophagy induction due to salt and starvation stress is NADPH-oxidase dependent (Liu et al., 2009), while autophagy induction due to other abiotic stresses, for example osmotic and ER stress, is NADPH- independent (Liu et al., 2009; Liu et al., 2012). Since GSH is involved in scavenging ROS, we hypothesized that inhibition of GSH synthesis will increase autophagy activity during stress conditions by altering the redox state, increasing the amount of stress-related ROS, and that adding exogenous GSH will be reducing the amount of ROS produced during stress, restoring the autophagy activity to control levels. To test our hypothesis, (1) GSH synthesis was inhibited with BSO in transgenic seedlings expressing green fluorescent protein (GFP)-AUTOPHAGY-RELATED 8e (ATG8e) (Xiong et al., 2007) and (2) exogenous GSH was added to GFP-ATG8e seedlings. ATG8e is an autophagosome membrane protein and is used as an autophagosome marker for quantifying autophagy activity (Contento et al., 2005; Floyd et al., 2015; Pu and Bassham, 2016).

3.3.2.1 GSH reduces autophagy activation during osmotic stress

To induce osmotic stress, seedlings were treated with 350 mM mannitol for 6 hours (Liu et al., 2009). To inhibit GSH synthesis during osmotic stress, seedlings were co-treated with BSO and mannitol for 6 hours. To assess the effects of exogenous GSH during osmotic stress, seedlings were co-treated with GSH and mannitol for 6 hours. Finally, seedlings were also co-treated with BSO, GSH and mannitol to determine the effect of adding exogenous

GSH when GSH synthesis is inhibited. After stress and/or chemical treatment the seedlings were imaged by fluorescence microscopy. Pictures were taken and autophagosomes were counted as a measure for autophagy activity. Under unstressed conditions, the addition of BSO, GSH or both had no effect on autophagy activity (Figure 2A). Autophagy was activated after osmotic stress treatment. Under osmotic stress conditions, autophagy activity was not affected by BSO treatment when compared to control, while addition of GSH resulted in reduction of autophagy activity (Figure 2A). Addition of exogenous GSH while inhibiting GSH synthesis with BSO under osmotic stress resulted in decreased autophagy activity (Figure 2A). Together, these results suggest that under osmotic stress, GSH reduces autophagy activation, possibly by a decrease in ROS.

3.3.2.2 GSH does not affect autophagy activation during oxidative stress

Next, we investigated the effect of BSO and exogenous GSH during oxidative stress. To induce oxidative stress, we treated seedlings with 10 mM hydrogen peroxide for 2 hours (Xiong et al., 2007). Treatments with BSO and GSH were performed as described above. Under unstressed conditions, the addition of BSO, GSH or both had no effect on autophagy activity. Autophagy was activated after oxidative stress treatment (Figure 2B). Under oxidative stress conditions autophagy activity was not affected by BSO or GSH treatment when compared to the control (Figure 2B). Inhibition of GSH synthesis by BSO and addition of exogenous GSH together under oxidative stress did not affect autophagy induction (Figure 2B). These results suggest that during oxidative stress, GSH has no effect in autophagy activation, possibly because the amount of ROS present cannot be quenched completely by GSH, resulting in activation of autophagy.

3.3.2.3 BSO blocks autophagy induced by salt stress and nutrient starvation

During salt stress and nutrient starvation autophagy induction depends on NADPH oxidase. Inhibition of NADPH-oxidase by the chemical inhibitors diphenylene iodinium (DPI) or imidazole (Jiang and Zhang, 2002) resulted in inhibition of autophagy during salt stress and nutrient starvation (Liu et al., 2009), suggesting that autophagy activation is triggered by the ROS produced by NADPH oxidase. Next, we investigated the effect of BSO and exogenous GSH during salt stress and sucrose starvation. To induce salt stress, we treated seedlings with 160 mM of sodium chloride for 6 hours (Liu et al., 2009). To induce starvation stress, seedlings were transferred to medium without sucrose and grown in the dark for 4 days. After 4 days, the seedlings were transferred to half-strength MS liquid medium without sucrose and treated with BSO, GSH or both as described above. Under unstressed conditions, the addition of BSO, GSH or both had no effect on autophagy activity. Autophagy was induced after salt treatment (Figure 2C) or sucrose starvation (Figure 2D). BSO treatment inhibited autophagy induction by salt stress and sucrose starvation, while autophagy activation was not affected by addition of GSH (Figure 2C and 2D). Addition of exogenous GSH while inhibiting GSH synthesis with BSO under salt stress or sucrose starvation rescued the autophagy inhibition (Figure 2C and 2D). Together, these results suggest that glutathione synthesis is important for autophagy activation under salt and starvation conditions, while during osmotic and oxidative stress glutathione synthesis is not essential for autophagy activation.

Next, we wanted to confirm that the decreased number of autophagosomes observed upon BSO treatment under salt stress and nutrient starvation was due to lack of autophagosome formation and not from acceleration of autophagosome degradation. We used salt treatment as a representative stress and treated 7-day-old GFP-ATG8e seedlings

with 1 μM concanamycin A (ConA) for 6 hours to block degradation of autophagosomes (Dröse et al., 1993; Klionsky et al., 2016). Salt stress and chemical treatments were performed as above. After treatment, seedlings were imaged by confocal microscopy. If BSO inhibits autophagosome formation, then after ConA treatment, no autophagosomes would accumulate during stress conditions. If BSO accelerates autophagosome degradation, then ConA treatment would result in accumulation of autophagosomes under stress conditions. Under unstressed conditions, the addition of BSO, GSH or both had no effect on the accumulation of autophagosomes. After salt stress treatment autophagy was activated as shown by the accumulation of GFP-ATG8e puncta (Figure 3). Under salt stress conditions autophagy was inhibited after BSO treatment, as shown by the lack of accumulation of GFP-ATG8e puncta (Figure 3). Addition of GSH under salt stress did not affect the accumulation of autophagosomes (Figure 3). Addition of exogenous GSH after inhibition by BSO restores the accumulation of autophagosomes (Figure 3). These results indicate that inhibition of GSH synthesis by BSO during salt stress reduces the number of autophagosomes observed by blocking autophagosome formation, rather than by accelerating autophagosome degradation.

3.3.3 Autophagy activation under salt and starvation stress is inhibited in the GSH-deficient mutant *cad2-1*

As an independent approach to confirm that the effect of BSO on autophagy is via inhibition of GSH synthesis, a glutathione-deficient mutant, *cad2-1* (Howden et al., 1995), was analyzed for autophagy under abiotic stress conditions. The *cad2-1* mutant is deficient in the first enzyme in the pathway of GSH biosynthesis, γ-glutamylcysteine (Cobbett et al., 1998), and it has only 15-30% of the GSH content of wild-type (Howden et al., 1995).

Seven-day-old wild-type and *cad2-1* seedlings were subjected to abiotic stress (osmotic, oxidative, salt or starvation stress) as described above and autophagosomes were labeled with the autophagosome-selective dye monodansylcadaverine (MDC) (Contento et al., 2005). After MDC staining, the seedlings were imaged by fluorescence microscopy. Pictures were taken and fluorescent puncta were counted as a measure of autophagy activity (Floyd et al., 2015). Under control conditions, *cad2-1* seedlings had a basal level of autophagy similar to wild-type, and autophagy activity was induced in *cad2-1* mutant after osmotic (Figure 4A) or oxidative stress (Figure 4C). By contrast, in *cad2-1,* autophagy was not induced after salt stress (Figure 4E) and starvation stress (Figure 4G) when compared to wild-type.

To confirm the MDC staining results, protoplasts from 4- to 6-week-old wild-type and *cad2-1* plants were transiently transformed with a GFP-ATG8e construct to assess autophagy activity (Contento et al., 2005). After transformation, protoplasts were incubated in the dark overnight to allow expression. After that, protoplasts were treated with abiotic stress and chemical treatments with the same concentrations as described above with the exception of sucrose starvation. For sucrose starvation, 1% sucrose was added to the control samples after transformation and protoplasts were incubated for 2 days in darkness (Soto-Burgos and Bassham, 2017). Under control conditions, *cad2-1* protoplasts had a basal level of autophagy similar to wild-type, and autophagy activity was induced in the *cad2-1* mutant after osmotic (Figure 4B) or oxidative stress (Figure 4D). Autophagy in *cad2-1* was not induced after salt stress (Figure 4F) when compared to unstressed *cad2-1*. During starvation stress autophagy was not activated in the *cad2-1* mutant when compared to wild-type (Figure 4H).

Wild-type and *cad2-1* seedlings and protoplasts were treated with exogenous GSH, as previously described, under control conditions and abiotic stresses to determine if the autophagy phenotype can be rescued. Upon adding exogenous GSH, the induction of autophagy was rescued in the *cad2-1* mutant under salt stress and nutrient starvation (Figure 4F and 4H). Together, these results demonstrate that GSH is required for the activation of autophagy under salt and starvation stress, the same conditions in which autophagy activation is dependent on production of ROS by NADPH oxidase.

3.3.4 Exogenous GSH rescues autophagy induction by salt stress in the absence of ROS

Autophagy induction by salt stress and nutrient starvation is NADPH oxidase-dependent, and autophagy activation is triggered by the ROS produced by NADPH oxidase (Liu et al., 2009). Since GSH is required for activation of autophagy under salt stress and nutrient starvation, we wanted to determine if ROS is required for the effect of GSH to signal autophagy activation. To address this question, we quenched the ROS using ascorbic acid (ASC) in the presence or absence of GSH, with salt stress as a representative stress. Seven-day-old wild-type and *cad2-1* seedlings were treated with salt and GSH as described before. For ASC treatment, seedlings were transferred to half-strength MS liquid medium supplemented with 2 mM ascorbic acid for 4 hours. After treatment, the seedlings were stained with MDC, followed by imaging by fluorescence microscopy. Pictures were taken, and puncta were counted as a measure for autophagy activity. Under control conditions *cad2-1* seedlings have a basal level of autophagy similar to wild-type, and autophagy activity was not induced in the *cad2-1* mutant after salt stress (Figure 5A). Autophagy in *cad2-1* was induced during salt stress after exogenous GSH was added (Figure 5A). Addition of ascorbic acid during salt stress blocked autophagy activation in wild-type and *cad2-1* seedlings

(Figure 5A). Autophagy was partially activated in wild-type and *cad2-1* seedlings during salt stress in the presence of GSH and ascorbic acid (Figure 5A).

To confirm these results, protoplasts from 4- to 6-week-old wild-type and *cad2-1* plants were transiently transformed with a GFP-ATG8e construct to assess autophagy activity (Contento et al., 2005) as described above. After that, protoplasts were treated with salt stress and chemical treatments as described before. Under control conditions, *cad2-1* protoplasts have a basal level of autophagy similar to wild-type, and autophagy activity was not induced in *cad2-1* mutant after salt stress (Figure 5B). Autophagy in *cad2-1* was induced during salt stress in the presence of GSH when compared to *cad2-1* without GSH (Figure 5B). Addition of ascorbic acid during salt stress blocked autophagy activity in wild-type and *cad2-1* seedlings (Figure 5B). Autophagy was partially activated in wild-type and *cad2-1* seedlings during salt stress in the presence of GSH and ascorbic acid (Figure 5B). These results confirm that the induction of autophagy during salt stress is due to the ROS generated, but also demonstrate that GSH works independently of ROS to activate autophagy during salt stress.

3.3.5 GSH activates autophagy through the SnRK1 complex

Previous research suggested that the target of rapamycin (TOR), a negative regulator of autophagy, can act either downstream of NADPH oxidase or in an NADPH oxidase-independent pathway (Liu and Bassham, 2010). Since autophagy during salt and starvation stress is regulated in a NADPH oxidase-dependent manner (Liu et al., 2009) and these stresses were the only ones that required GSH for autophagy activation, we investigated if GSH works upstream of known regulators of autophagy. We hypothesized that regulation of autophagy by GSH acts upstream of the TOR complex. The TOR complex is the main

component of the TOR signaling pathway and it consists of the TOR serine/threonine kinase catalytic subunit (Menand et al., 2002), RAPTOR (Anderson et al., 2005; Deprost et al., 2005), which presents substrates to TOR for phosphorylation (Hara et al., 2002) and LST8, which maintains the stability of the complex (Moreau et al., 2012). There are two *RAPTOR* genes in *Arabidopsis, RAPTOR1A* and *RAPTOR1B* and disruption of the *RAPTOR1B* gene results in constitutive autophagy (Menand et al., 2002; Pu et al., 2017).

To test this hypothesis, 7-day-old WT and *raptor1b* seedlings were treated with BSO, GSH or BSO plus GSH as described above. Autophagosomes were labeled with MDC and quantification of autophagy activity, autophagosomes per frame, was determined. Under control conditions autophagy in the *raptor1b* mutant is active (Figure 6A). Inhibiting GSH synthesis or adding exogenous GSH had no effect on the constitutive autophagy in the *raptor1b* mutant (Figure 6A). To confirm these results, protoplasts from 4- to 6-week-old wild-type and *raptor1b* plants were transiently transformed with a GFP-ATG8e construct to assess autophagy activity (Contento et al., 2005) as described before. After that, protoplasts were treated with chemical treatments as previously described. Consistent with the previous results, autophagy was active in the *raptor1b* mutant under control conditions, and inhibiting GSH synthesis or adding exogenous GSH had no effect on the autophagy activity of the *raptor1b* mutant (Figure 6B). These results suggest that regulation of autophagy by GSH acts upstream of TOR.

Next, we tested the role of GSH in the regulation of autophagy upstream of TOR. The sucrose non-fermenting 1 (SnRK1) complex is a positive regulator of autophagy (Chen et al., 2017; Soto-Burgos and Bassham, 2017) and acts upstream of TOR (Soto-Burgos and Bassham, 2017). The SnRK1 complex has a α-catalytic subunit (KIN10) and regulatory

subunits (β and γ) (Crozet et al., 2014). Plants with overexpression of KIN10 have increased autophagy, while *kin10* mutants cannot activate autophagy during most abiotic stresses (Soto-Burgos and Bassham, 2017). Seven-day-old WT and KIN10 overexpression seedlings were treated with BSO, GSH or BSO plus GSH as described above. Autophagosomes were labeled with MDC and fluorescent puncta were counted. Under control conditions autophagy is active in KIN10 overexpression seedlings (Figure 6C), but inhibition of GSH synthesis by BSO blocked this autophagy (Figure 6C). Addition of exogenous GSH had no effect on autophagy activity in KIN10 overexpression seedlings (Figure 6C). Addition of exogenous GSH while inhibiting GSH synthesis with BSO rescued the autophagy activity of KIN10 overexpression (Figure 6C). To confirm these results, protoplasts from 4- to 6-week-old wild-type and KIN10 overexpression plants were transiently transformed with a GFP-ATG8e construct to assess autophagy activity as described before. After that, protoplasts were treated with BSO, GSH or BSO plus GSH as previously described. Consistent with the previous results, autophagy was active in KIN10 overexpression plants under control conditions, and inhibiting GSH synthesis blocked this autophagy activation (Figure 6D). Addition of exogenous GSH had no effect on the autophagy activity of KIN10 overexpression (Figure 6D). Addition of exogenous GSH while inhibiting GSH synthesis with BSO rescued the autophagy activity of KIN10 overexpression. These results suggest that GSH acts via the SnRK1 complex in the activation of autophagy, possibly by controlling SnRK1 activity. Together, these results suggest that autophagy is regulated by GSH upstream of TOR, most likely through the SnRK1 complex followed by NADPH-oxidase regulation during salt stress and sucrose starvation.

3.4 Discussion

GSH is an important antioxidant that maintain cellular redox status and prevents cellular damage (Aquilano et al., 2014). In mammals, GSH has been shown to decrease autophagy during nutrient starvation (Desideri et al., 2012). In yeast, a decrease in GSH levels have been shown to increase the selective autophagy of mitochondria, mitophagy (Deffieu et al., 2009). To date, there has not been a report for a connection between autophagy and GSH in plants. This work focusses on understanding the role of GSH in the activation of autophagy under different abiotic stresses in *Arabidopsis.* Our results demonstrate that GSH is required for the activation of autophagy under salt stress and nutrient starvation, but not during oxidative or osmotic stress. Also, our results suggest that this regulation occurs via the SnRK1 complex, possibly by its activation.

The enzyme NADPH-oxidase produces ROS under various abiotic and biotic stresses including salt stress and nutrient starvation (Jiang and Zhang, 2002; Liu et al., 2009). The scavenging of radicals is one of the many roles of GSH in the cell (Aquilano et al., 2014). Here, we found that inhibition of GSH, either chemically or genetically, blocked autophagy induction by salt stress and sucrose starvation, while autophagy activation was not affected during oxidative and osmotic stresses. These findings suggest that the scavenging of ROS by GSH during abiotic stress is not the primary role of GSH during autophagy. Addition of exogenous GSH while inhibiting GSH synthesis with BSO under salt stress or sucrose starvation rescued the autophagy activation, indicating that GSH has a role in autophagy activation. In the case of osmotic stress, a reduction of autophagy activity was observed after addition of exogenous GSH under stress in GFP-ATG8e seedlings, while in wild-type under the same conditions we observed full autophagy activation. In both genotypes, activation of

autophagy still occurs but at different levels. A possibility is that the overexpression of ATG8 in the GFP-ATG8e seedlings is causing some differences in autophagy in response to osmotic stress. Another possibility is that an unaccounted environmental difference occurred while conducting the experiment. Further testing is necessary to distinguish between the possibilities. We found that under salt stress conditions autophagy induction was inhibited after BSO treatment, even after ConA treatment was used for blocking autophagosome degradation and addition of exogenous GSH after inhibition by BSO restores the autophagy induction. This confirmed that GSH is required to activate autophagosome formation during salt stress. For the results observed during starvation and salt stress, one possibility is that GSH is involved in the signaling pathway controlling autophagosome formation.

In animal cells, autophagy is regulated via superoxide production (Chen et al., 2009) and by the inactivation of ATG4 by ROS during starvation, thus activating autophagy (Scherz-Shouval et al., 2007). We found that quenching of ROS using ascorbic acid in the presence or decrease of endogenous GSH resulted in autophagy inhibition upon salt stress. Addition of exogenous GSH partially activated autophagy in wild-type and *cad2-1* seedlings during salt stress plus ascorbic acid. These results demonstrate that even when ROS is quenched, GSH is still required to activate autophagy, therefore GSH works independently of ROS to activate autophagy during salt stress. These results are consistent with reports in animal cells, that demonstrate that GSH regulation of autophagy is not due to scavenging of ROS during starvation (Desideri et al., 2012).

Protein S-glutathionylation consists of the reaction of glutathione with the free thiol (SH) in certain cysteine (Cys) residues of proteins. GSH covalently attaches to oxidant-induced protein thiyl radicals (RS•), a reactive sulphur species formed after ROS oxidation

of Cys–SH group, resulting in protein S-glutathionylation (Popov, 2014). In mammals, AMPK has been reported to be activated by S-glutathionylation of Cys299 and Cys304 in the catalytic subunit upon exposure to hydrogen peroxide (Zmijewski et al., 2010). One possibility is that GSH post-translationally modifies KIN10 to activate it like its mammalian ortholog AMPK, to regulate autophagy. Although, S-glutathionylation of KIN10 cannot be ruled out, the fact that GSH can rescue the activation of autophagy even with ROS quenched, suggest that is unlikely that regulation of autophagy by glutathione is due to S-glutathionylation of KIN10.

TOR, a negative regulator of autophagy, and the SnRK1 complex, a positive regulator of autophagy, were possible candidates for GSH regulation. TOR can act either downstream of NADPH oxidase or in an NADPH oxidase-independent pathway (Liu and Bassham, 2010). Autophagy during salt and starvation stress is regulated in a NADPH- oxidase dependent manner (Liu et al., 2009) and these stresses were the only ones that required GSH for autophagy activation. Inhibition of GSH synthesis or adding exogenous GSH had no effect on the constitutive autophagy in the *raptor1b* mutant, suggesting that regulation of autophagy by GSH acts upstream of TOR. SnRK1 acts upstream of TOR in the regulation of autophagy (Soto-Burgos and Bassham, 2017). Inhibition of GSH synthesis by BSO blocked the autophagy activity of KIN10 overexpression seedlings and addition of exogenous GSH while inhibiting GSH synthesis with BSO rescued the autophagy activity. These results suggest that GSH acts via the SnRK1 complex in the activation of autophagy. Recently, it has been shown in *Arabidopsis* that activation of SnRK1 is regulated by the redox status *in vitro* (Wurzinger et al., 2017). KIN10 activity increased as the conditions became more reduced or by addition of GSH. Reducing conditions or addition of GSH promoted KIN10

phosphorylation by upstream kinases. This indicates that GSH keeps KIN10 in a reducing state, resulting in full intrinsic KIN10 kinase activity as well as phosphorylation of KIN10 being by upstream kinases. Since autophagy during salt stress can be activated in the presence of GSH, even when ROS are quenched, it is probable that the environment is in a reducing state leading to activation of the SnRK1 complex.

Taking all these results together, we propose a possible model for the regulation of autophagy by GSH during salt stress and nutrient starvation. Upon salt stress or nutrient starvation, the SnRK1 can be activated by GSH or by the ROS produced by NADPH-oxidase. After SnRK1 activation, autophagy can be regulated via TOR inactivation or by activation of the ATG1 complex (Figure 7). In conclusion, we have identified GSH as a regulator of autophagy during salt stress and nutrient starvation, acting upstream of TOR possibly by activation of SnRK1. Further work is necessary to determine precisely if SnRK1 activity is affected by GSH *in vivo* and if it does, by which mechanism GSH regulates SnRK1 activity to activate autophagy.

3.5 Methods

3.5.1 Plant materials and growth conditions

Wild-type (Col-0), GFP-ATG8e (Xiong et al., 2007), *cad2-1* (Howden et al., 1995), KIN10 OX (Baena-González et al., 2007), *raptor1b* (SALK_078159) (Menand et al., 2002), and *kin10* (SALK_127939C) (Alonso et al., 2003; Fragoso et al., 2009) *Arabidopsis thaliana* seeds were surface-sterilized with 33% (v/v) bleach, 0.1% (v/v) Triton X-100 solution for 20 minutes, washed 5 times with sterile water, and kept at 4°C for at least 2 days in the dark. Plants were grown on half-strength MS medium (Murashige-Skoog with vitamins mixture

[Caisson, MSP09], 2.4 mM MES [pH 5.7], 1% sucrose, 0.6% Phytoblend agar) or in soil under long day conditions (16 h light and 8 h dark) at 22°C.

3.5.2 Stress and chemical treatments

For osmotic stress, 7-day-old seedlings were transferred to half-strength MS liquid medium with 350 mM mannitol for 6 hours (Liu et al., 2009). For oxidative stress, 7-day-old seedlings were transferred to half-strength MS liquid medium supplemented with 10 mM hydrogen peroxide for 2 hours (Xiong et al., 2007). For salt stress, 7-day-old seedlings were transferred to half-strength MS liquid medium with 160 mM NaCl for 6 hours (Liu et al., 2009). For starvation stress, 7-day-old seedlings were transferred to half-strength MS plates with sucrose or lacking sucrose for 4 days and were incubated in the dark.

For buthionine sulfoximine (BSO) treatment, 7-day old seedlings were transferred to half-strength MS liquid medium supplemented with 1 mM BSO [Sigma B2515] for 6 hours (Xiang and Oliver, 1998). For treatment in the presence of stress, seedlings were subjected to the abiotic stress conditions as described above and BSO was added to a final concentration of 1 mM. Seedlings under starvation stress were transferred after the 4 days of starvation to half-strength MS liquid medium lacking sucrose and supplemented with 1 mM BSO for 6 hours in the dark.

For glutathione (GSH) treatment, 7-day old seedlings were transferred to half-strength MS liquid medium supplemented with 2 mM GSH [Sigma G6529] for 6 hours (Xiang and Oliver, 1998). For treatment in the presence of stress, seedlings were subjected to the abiotic stress conditions as described above and GSH was added to a final concentration of 2 mM. Seedlings under starvation stress were transferred after the 4 days of starvation to

half-strength MS liquid medium lacking sucrose and supplemented with 2 mM GSH for 6 hours in the dark.

For ascorbic acid treatment, 7-day old seedlings were transferred to half-strength MS liquid medium supplemented with 2 mM ascorbic acid [Fisher A61] for 4 hours (Liu and Bassham, 2010). For treatment in the presence of salt stress, seedlings were subjected to the salt stress conditions as described above and ascorbic acid was added to a final concentration of 2 mM for the last 4 hours of treatment.

For Concanamycin A (ConA) treatment, 7-day old GFP-ATG8e seedlings were transferred to half-strength MS liquid medium supplemented with 1 μM ConA [Sigma C9705] or DMSO, with or without salt stress for 6 hours.

3.5.3 Transient transformation of leaf protoplasts

Leaf protoplasts were prepared from 4- to 6-week-old *Arabidopsis* plants and 20 μg of plasmid DNA encoding GFP-ATG8e was used for each transformation as described (Sheen, 2002). Protoplasts were incubated in darkness overnight at room temperature, with 40 rpm orbital shaking, followed by stress and/or chemical treatment as described above, with the exception of sucrose starvation. For sucrose starvation, 1% sucrose was added to the control samples after transformation and protoplasts were incubated for 2 days in darkness. Autophagosomes labeled with GFP-ATG8e were counted under a Nikon E200 fluorescence microscope. A protoplast was considered to have active autophagy if 3 or more GFP-ATG8e-labeled autophagosomes were detected (Yang et al., 2016).

3.5.4 GSH extraction and HPLC analysis

Wild-type *Arabidopsis* 10-day-old seedlings grown on MS medium were transferred to MS liquid medium with 1 mM BSO or with 1 mM BSO plus 2 mM GSH and incubated for 6 hours. Seedlings were ground and for every 40 mg of fresh weight of tissue, 160 µL of 0.1 M hydrochloric acid with 1 mM EDTA were added, followed by 5 minutes in an ice bath. Samples were centrifuged at 12,000 xg for 10 minutes at 4°C. 40 µL of the supernatant was transferred into 28 µL of reaction buffer (10 µM N-cyclohexyl-2-aminoethanesulfonic acid (CHES) (pH 9.3), 40 nM dithiothreitol, 120 nM monobromobimane) followed by a 15-minute incubation at room temperature in the dark. After incubation, 160 µL of 10% acetic acid was added to stop the reaction. The solution was kept on ice for 5 minutes, followed by centrifugation at 10,000 xg for 30 minutes at 4°C. The supernatant was transferred to an amber HPLC vial. For analysis, 100 µL of sample was injected into the HPLC and quantification was performed fluorometrically after separation by reversed-phase HPLC (Xiang and Oliver, 1998).

3.5.5 MDC staining and microscopy

Arabidopsis seedlings were stained with 0.05 mM monodansylcadaverine (MDC) dissolved in phosphate-buffered saline (PBS) for 10 minutes, followed by 3 washes with PBS (Contento et al., 2005). Seedlings were observed using a Zeiss AxioImager microscope, using a X40 objective and a 4',6-diamidino-2-phenylindole (DAPI) filter. GFP fluorescence was imaged using a X40 objective and a fluorescein isothiocyanate (FITC) filter.

Confocal microscopy was performed using a Leica SP5 confocal laser scanning microscope with a X63 oil immersion objective. The excitation and emission wavelengths for

GFP were 488 and 507 nm respectively. All the microscopy was performed at the Roy J. Carver High Resolution Microcopy Facility at Iowa State University.

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3.7 Figures

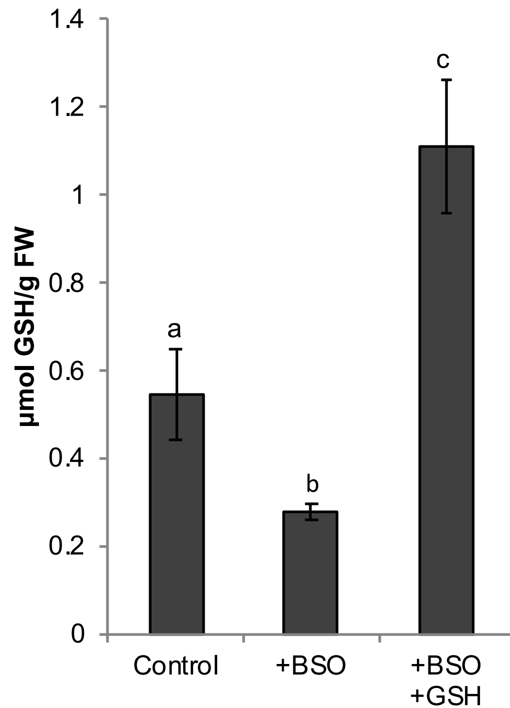

Figure 1. BSO reduces GSH content in *Arabidopsis thaliana*

Ten-day-old wild-type seedlings were transferred to MS liquid media supplemented with 1 mM BSO, 1 mM BSO plus 2 mM GSH, or an equivalent amount of water for 6 h, followed by extraction of GSH and quantification by HPLC. Error bars represent standard error. Different letters denote statistical significance, $p<0.05$, t-test.

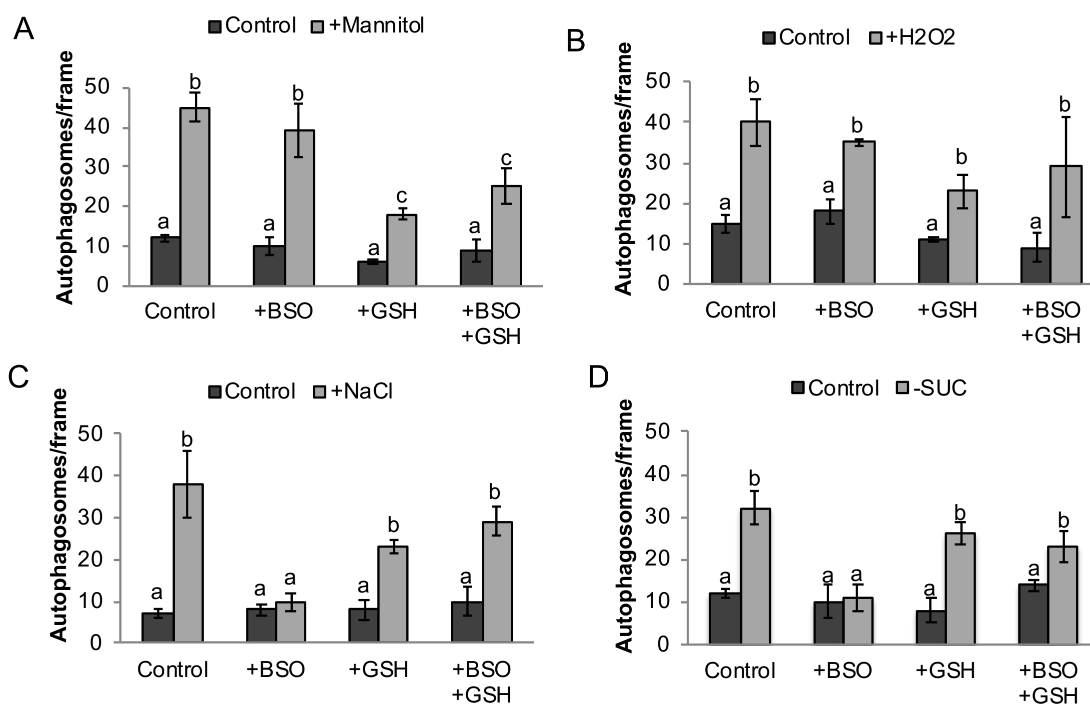

Figure 2. Inhibition of GSH synthesis reduces autophagy activation during salt stress or nutrient starvation

Seven-day-old GFP-ATG8e seedlings were transferred to ½ MS liquid medium supplemented with 1 mM BSO, 2 mM GSH or 1 mM BSO plus 2 mM GSH for 6 hours as control, or liquid medium supplemented with 350 mM mannitol and 1 mM BSO, 2 mM GSH or 1 mM BSO plus 2 mM GSH for 6 hours **(A)**, liquid medium supplemented with 1 mM BSO, 2 mM GSH or 1 mM BSO plus 2 mM GSH for 6 hours and 10 mM hydrogen peroxide added for the last 2 hours **(B)**, liquid medium supplemented with 160 mM NaCl and 1 mM BSO, 2 mM GSH or 1 mM BSO plus 2 mM GSH for 6 hours **(C)**, or ½ MS plates lacking sucrose for 4 days in the dark followed by 1 mM BSO, 2 mM GSH or 1 mM BSO plus 2 mM GSH treatment in liquid medium for 6 hours **(D)**. Autophagosomes were imaged using epifluorescence microscopy and counted. Different letters denote statistical significance for three biological replicates with at least 9 frames per replicate, $p<0.05$, t-test. Error bars show standard error.

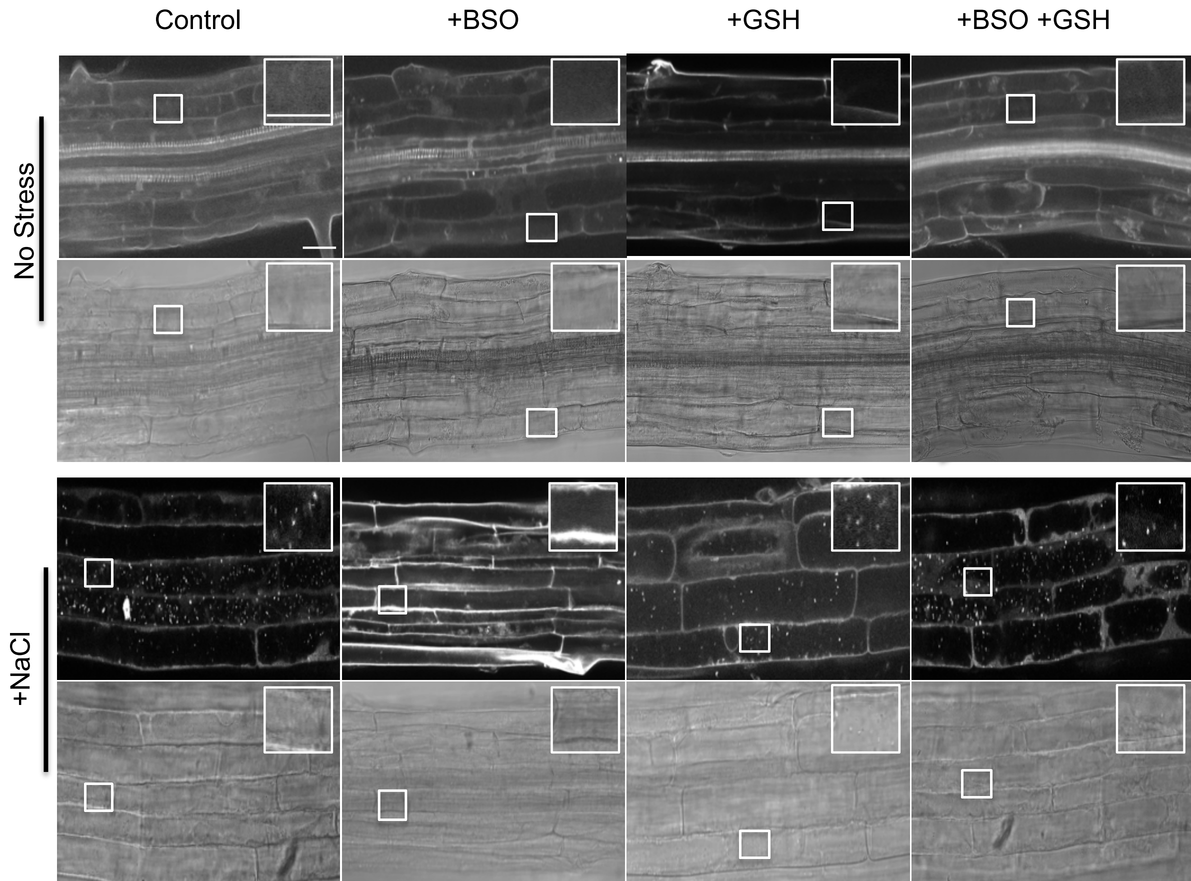

Figure 3. Inhibition of GSH synthesis blocks autophagosome formation during salt stress

Seven-day-old GFP-ATG8e seedlings were transferred to ½ MS liquid medium with 1 μM concanamycin A and 1 mM BSO, 2 mM GSH or 1 mM BSO plus 2 mM GSH for 6 hours as control, or ½ MS liquid medium with 1 μM concanamycin A with 160 mM NaCl and 1 mM BSO, 2 mM GSH or 1 mM BSO plus 2 mM GSH for 6 hours. Autophagosomes were imaged using confocal microscopy. The insets show enlargements of the indicated boxes. Scale bars = 30 μm.

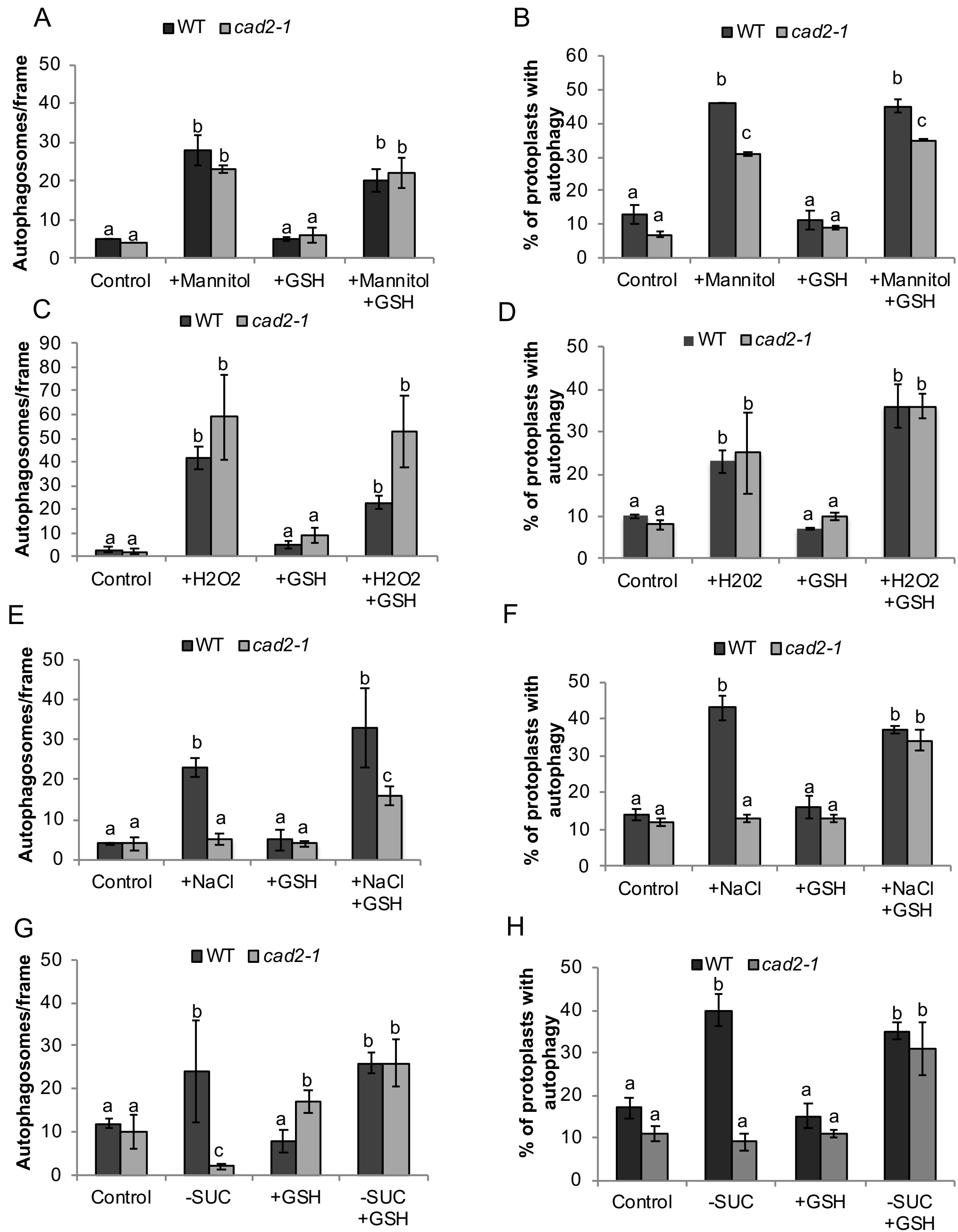

Figure 4. The GSH-deficient *cad2-1* mutant has decreased autophagy under salt or starvation stress

(A, C, E, G) Seven-day-old WT and *cad2-1* seedlings were transferred to ½ MS liquid medium supplemented with 2 mM GSH for 6 hours as control, or liquid medium supplemented with 350 mM mannitol or 350 mM mannitol plus 2 mM GSH for 6 hours **(A)**, liquid medium supplemented with 10 mM hydrogen peroxide or 2 mM GSH for 6 hours and 10 mM hydrogen peroxide added for the last 2 hours **(C)**, liquid medium supplemented with 160 mM NaCl or 160 mM NaCl plus 2 mM GSH for 6 hours **(E)**, or ½ MS plates lacking sucrose for 4 days in the dark followed by 2 mM GSH treatment in liquid medium for 6 hours **(G)**. Seedlings were stained with MDC and autophagosomes counted. Different letters denote statistical significance for three biological replicates with at least 9 frames per replicate, $p<0.05$, t-test.

(B, D, F, H) WT and *cad2-1* protoplasts were transiently transformed with the autophagy marker GFP-ATG8e, incubated overnight to allow expression, and then the protoplast solution was supplemented with 2 mM GSH for 6 hours as control, or with 350 mM mannitol or 350 mM mannitol plus 2 mM GSH for 6 hours **(B)**, 10 mM hydrogen peroxide or 2 mM GSH for 6 hours and 10 mM hydrogen peroxide added for the last 2 hours **(D)**, with 160 mM NaCl or 160 mM NaCl plus 2 mM GSH for 6 hours **(F)**, or incubated plus or minus 1% sucrose for 48 hours followed by addition of 2 mM GSH for 6 hours **(H)**. Autophagosomes were visualized by epifluorescence microscopy and the percentage of protoplasts with active autophagy determined. Different letters denote statistical significance for three biological replicates with 100 protoplasts for each sample per replicate, $p<0.05$, t-test. Error bars indicate standard error.

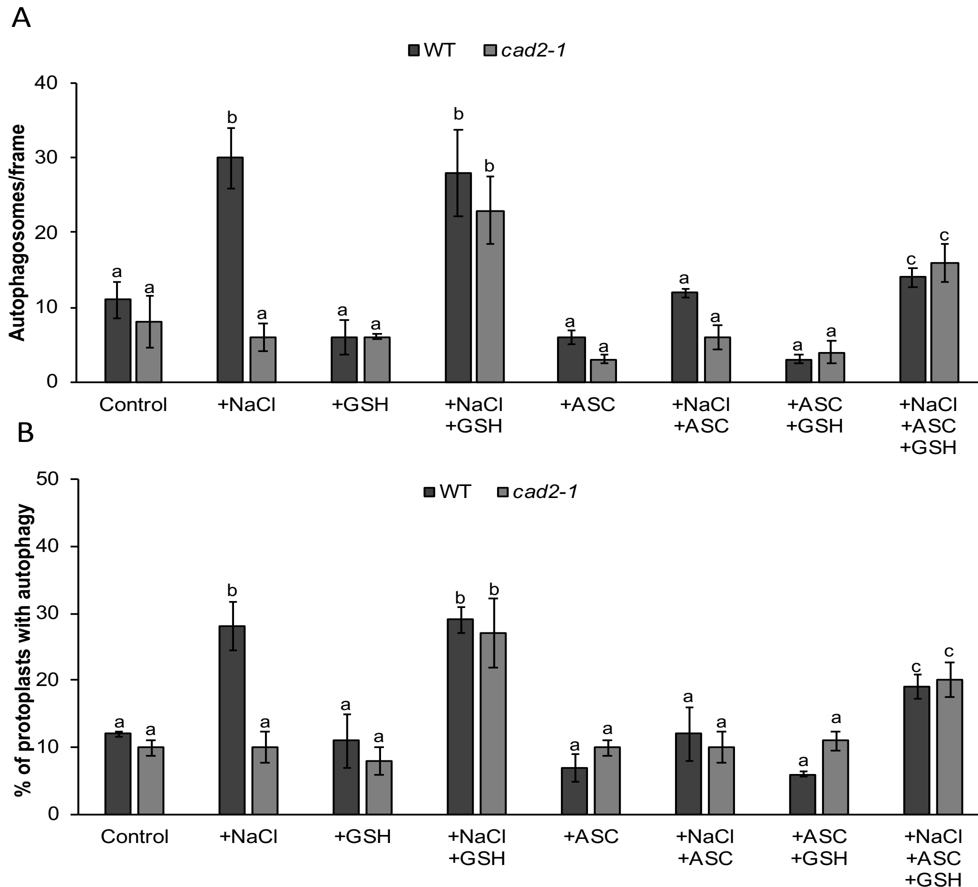

Figure 5. Activation of autophagy by GSH does not require ROS

(A) Seven-day-old WT and *cad2-1* seedlings were transferred to ½ MS liquid medium supplemented with 2 mM GSH for 6 hours, 2 mM ascorbic acid (ASC) for 4 hours, or 2 mM GSH for 6 hours and 2 mM ASC for the last 4 hours as control; or liquid medium supplemented with 160 mM NaCl for 6 hours, 160 mM NaCl plus 2 mM GSH for 6 hours, 160 mM NaCl for 6 hours plus 2 mM ASC for the last 4 hours, or 160 mM NaCl plus 2 mM GSH for 6 hours and 2 mM ASC for the last 4 hours. Seedlings were stained with MDC and autophagosomes counted. Different letters denote statistical significance for three biological replicates with at least 9 frames per replicate, $p<0.05$, t-test. **(B)** WT and *cad2-1* protoplasts were transiently transformed with the autophagy marker GFP-ATG8e, incubated overnight to allow expression, and then the protoplast solution was supplemented with 2 mM GSH for 6 hours, 2 mM ascorbic acid (ASC) for 4 hours, or 2 mM GSH for 6 hours and 2 mM ASC for the last 4 hours as control; or liquid medium supplemented with 160 mM NaCl for 6 hours, 160 mM NaCl plus 2 mM GSH for 6 hours, 160 mM NaCl for 6 hours plus 2 mM ASC for the last 4 hours, or 160 mM NaCl plus 2 mM GSH for 6 hours and 2 mM ASC for the last 4 hours. Autophagosomes were visualized by epifluorescence microscopy and the percentage of protoplasts with active autophagy determined. Different letters denote statistical significance for three biological replicates with 100 protoplasts for each sample per replicate, $p<0.05$, t-test. Error bars indicate standard error.

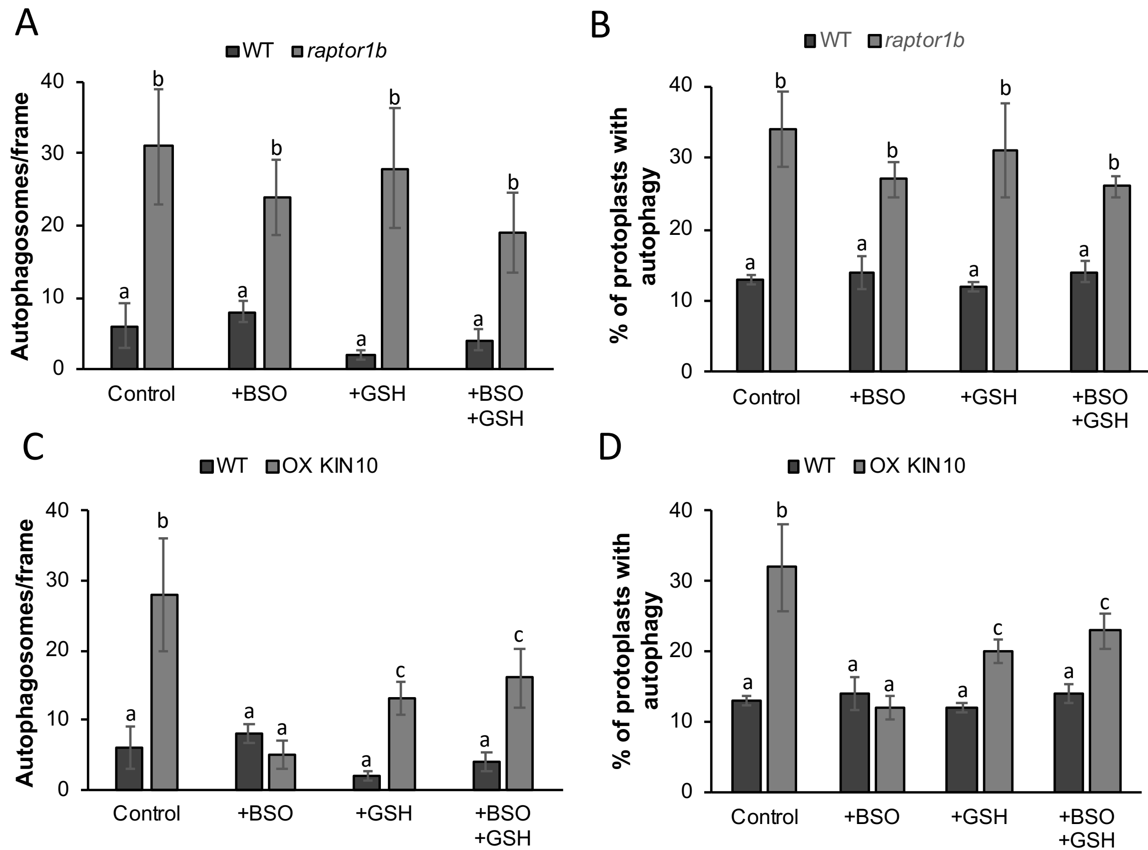

Figure 6. GSH activates autophagy via the SnRK1 complex

(A) Seven-day-old WT and *raptor1b* seedlings were transferred to ½ MS liquid medium supplemented with 1 mM BSO, 2 mM GSH or 1 mM BSO plus 2 mM GSH for 6 hours. Seedlings were stained with MDC and autophagosomes counted. **(B)** WT and *raptor1b* protoplasts were transiently transformed with the autophagy marker GFP-ATG8e, incubated overnight to allow expression, and then the protoplast solution was supplemented with 1 mM BSO, 2 mM GSH or 1 mM BSO plus 2 mM GSH for 6 hours. Autophagosomes were visualized by epifluorescence microscopy and the percentage of protoplasts with active autophagy determined. **(C)** Seven-day-old WT and KIN10 overexpression seedlings were transferred to ½ MS liquid medium supplemented with 1 mM BSO, 2 mM GSH or 1 mM BSO plus 2 mM GSH for 6 hours. Seedlings were stained with MDC and autophagosomes counted. **(D)** WT and KIN10 overexpression protoplasts were transiently transformed with the autophagy marker GFP-ATG8e, incubated overnight to allow expression, and then the protoplast solution was supplemented with 1 mM BSO, 2 mM GSH or 1 mM BSO plus 2 mM GSH for 6 hours. Autophagosomes were visualized by epifluorescence microscopy and the percentage of protoplasts with active autophagy determined. Different letters denote statistical significance for three biological replicates, $p<0.05$, t-test. Error bars indicate standard error.

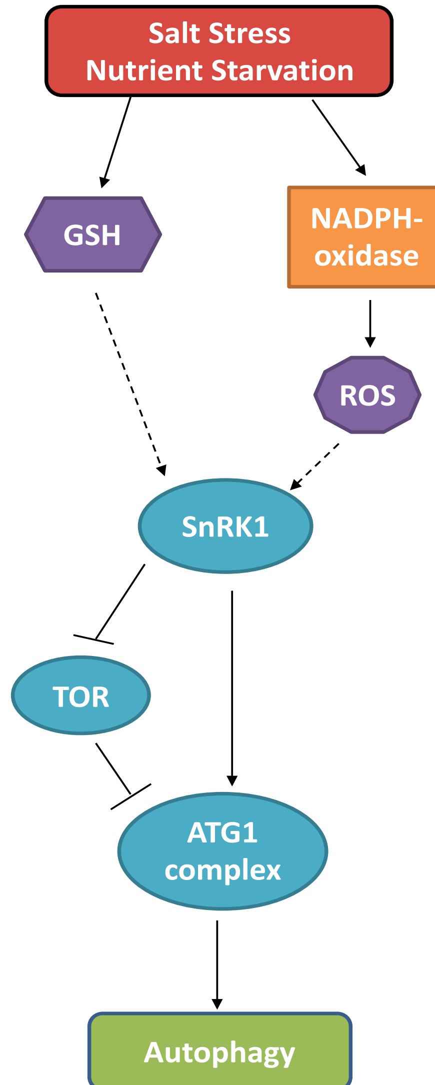

Figure 7. Model for the regulation of autophagy by GSH during salt stress and nutrient starvation

Upon salt stress and nutrient starvation, the SnRK1 complex can be activated by glutathione or by the ROS produced by NADPH-oxidase. Once active, the SnRK1 complex inhibits TOR, leading to activation of the ATG1 complex, which in turn activates autophagy. SnRK1 can also activate the ATG1 complex by phosphorylation of ATG1, leading to activation of autophagy. Solid arrows indicate pathways with experimental evidence, while dashed arrows indicate potential pathways that need future investigation.

CHAPTER 4. THE TOR SIGNALING PATHWAY IS INVOLVED IN THE ACTIVATION OF CONSTITUTIVE AUTOPHAGY IN *rns2-2* IN *Arabidopsis thaliana*

4.1 Abstract

Ribosomes account for most of the RNA in the cell and ribosome degradation plays a significant role in maintaining cellular homeostasis. The RNS2 ribonuclease and autophagy, a degradation process in which cells break down and recycle their cytoplasmic content, participate in ribosomal turnover in *Arabidopsis thaliana*. Disruption of RNS2 activity results in constitutive autophagy in which the autophagosomes contains RNA and ribosomes. In this study, I investigated possible pathways by which autophagy in the *rns2-2* mutant is activated. Using a chemical approach, I tested the SnRK1 complex, a positive regulator of autophagy, and the TOR signaling pathway, a negative regulator of autophagy. While wild-type plants had low basal autophagy activity in control conditions, the *rns2-2* mutant had increased autophagy under these conditions. Inhibition of the SnRK1 complex by trehalose-6-phosphate (T6P) did not inhibited the constitutive autophagy in *rns2-2* mutants, indicating that the activation of autophagy is independent of the SnRK1 complex. Activation of TOR kinase by auxin or the brassinosteroid, brassinolide (BL) resulted in the inhibition of the autophagy activity in the *rns2-2* mutant, indicating that the TOR signaling pathway is involved in the activation of autophagy in *rns2-2* mutant.

4.2 Introduction

Autophagy is a degradation pathway that cells use for recycling of cytoplasmic contents during certain development stages or under stress conditions (Soto-Burgos et al., 2018). Macroautophagy (hereafter referred to as autophagy) is characterized by the formation of double membrane vesicles, called autophagosomes, around the cargo targeted for

autophagy, through the action of autophagy-related (ATG) proteins. The autophagosome then delivers the cargo to the vacuole/lysosome for degradation.

The autophagy pathway is highly conserved among eukaryotes. To maintain homeostasis, cells have a basal level of autophagy under normal conditions (Soto-Burgos et al., 2018). In plants, autophagy is activated in response to stress conditions and during development. Some of the components of the pathways regulating plant autophagy have been identified: the ATG1 complex, Snf1-related protein kinase 1 (SnRK1) complex, target of rapamycin (TOR) and inositol-requiring enzyme-1 (IRE1) (Liu and Bassham, 2010; Suttangkakul et al., 2011; Liu et al., 2012; Chen et al., 2017; Soto-Burgos and Bassham, 2017). TOR acts as a negative regulator of the autophagy pathway, and it can regulate autophagy during starvation, salt and osmotic stress conditions (Pu et al., 2017). The SnRK1 complex positively regulates autophagy upstream of TOR (Soto-Burgos and Bassham, 2017). Autophagy can also be regulated in a NADPH oxidase dependent and -independent manner depending on the conditions (Liu et al., 2009; Liu et al., 2012) and this regulation possibly acts upstream of the TOR signaling pathway (Liu and Bassham, 2010).

In yeast, upon starvation ribosomes can be degraded by autophagy or ribophagy, a type of selective autophagy which targets ribosomes by recruiting components of the non-selective autophagy machinery, including the E1-like protein ATG7 and scaffold protein ATG17 (Lardeux and Mortimore, 1987; Kraft et al., 2008; Huang et al., 2015). rRNA turnover plays an important role in response to nutrient deficiency, but also for maintaining cellular homeostasis during normal conditions (Warner, 1999). In Arabidopsis, the vacuolar RNase RNS2 is a member of the RNase T2 family and is necessary for rRNA decay (MacIntosh et al., 2010; Hillwig et al., 2011). rRNAs have a longer half-life in Arabidopsis

mutants lacking RNS2 than in wild-type (Hillwig et al., 2011). rRNA accumulates in the vacuoles of rns2-2 mutants, and this accumulation is dependent on the core autophagy protein ATG5 but not ATG9 (Floyd et al., 2015), indicating different functions of ATG5 and ATG9 in the trafficking and turnover of rRNA in the vacuole by autophagy. Also, lack of RNS2 activity results in increased autophagy activity, suggesting that ribosomes or rRNA are targeted for vacuolar degradation through a selective autophagy process similar to ribophagy to maintain ribosomal turnover (MacIntosh and Bassham, 2011). rns2 mutants have elevated levels of reactive oxygen species (ROS) and inhibition of NADPH oxidase activity suppresses the constitutive autophagy in rns2-2 (Morriss et al., 2017), suggesting that ROS signaling controls autophagy in rns2-2 plants.

In this study, I used chemical treatments to determine through which mechanism the autophagy in the rns2-2 mutant is activated. Our results suggest that the TOR signaling pathway is involved in the activation of autophagy in the rns2-2 mutant, possibly due to the inactivation of the TOR kinase.

4.3 Results

4.3.1 Autophagy phenotype of *rns2-2* mutant is not regulated by SnRK1 complex

Loss of RNS2 activity in rns2-2 mutant leads to increased basal autophagy in Arabidopsis (Floyd et al., 2015). To investigate the mechanism by which a mutant deficient in RNS2 has active autophagy, we investigated the SnRK1 and TOR complexes, key regulators of the autophagy pathway in Arabidopsis (Liu and Bassham, 2010; Chen et al., 2017; Soto-Burgos and Bassham, 2017). Trehalose-6-phosphate (T6P) is a sugar that has been shown to inhibit the activity of the SnRK1 complex (Zhang et al., 2009). In

Arabidopsis, treatment with T6P during most abiotic stresses tested resulted in a block in autophagy activation (Soto-Burgos and Bassham, 2017). We therefore hypothesized that if the constitutive autophagy of rns2-2 is due to increased activity of the SnRK1 complex, then inhibition of SnRK1 in the rns2-2 mutant will result in loss of the constitutive autophagy phenotype.

To test this hypothesis, 7-day-old GFP-ATG8e- (Xiong et al., 2007) and rns2-2 GFP-ATG8e- (Floyd et al., 2015) expressing seedlings were transferred to half-strength MS liquid medium supplemented with or without 0.1 mM T6P for 3 hours. GFP-ATG8e is used as an autophagosome marker to measure autophagy activity by labeling autophagosomes. After treatment, seedlings were visualized by confocal microscopy (Figure 1A) or pictures were taken using an epifluorescence microscope for quantification. As expected, in control conditions, GFP-ATG8e seedlings had a low basal level of autophagy, while in rns2-2 GFP-ATG8e seedlings autophagy was active (Figure 1B). Inhibition of the SnRK1 complex by T6P had no effect on autophagy activity in GFP-ATG8e seedlings or rns2-2 GFP-ATG8e mutant when compared to the untreated control (Figure 1B). These results suggest that the autophagy activity observed in the rns2-2 mutant is not due to aberrant activation of the SnRK1 complex.

4.3.2 Activation of TOR complex results in inhibition of constitutive autophagy in *rns2-2* mutant

A second candidate for a factor that may lead to the constitutive autophagy in rns2-2 is the TOR kinase complex. The TOR complex is composed of the TOR kinase itself (Menand et al., 2002), RAPTOR, which presents substrates to TOR (Hara et al., 2002; Anderson et al., 2005; Deprost et al., 2005), and LST8 to stabilize the complex (Moreau et

al., 2012). In Arabidopsis, two RAPTOR genes have been identified, RAPTOR1A and RAPTOR1B (Anderson et al., 2005). While a TOR null mutant is embryo lethal (Menand et al., 2002), a mutation in RAPTOR1B results in plant growth and developmental defects (Anderson et al., 2005). A raptor1b mutant has constitutive autophagy, while transgenic lines that overexpress of TOR have a low basal level of autophagy same as wild-type (Pu et al., 2017). During nutrient starvation, salt stress and osmotic stress, autophagy is induced in wild-type plants, while plants overexpressing TOR failed to induce autophagy activity under those conditions (Pu et al., 2017). Activation of TOR kinase by auxin (Schepetilnikov et al., 2017) results in autophagy repression induced by nutrient starvation, salt stress and osmotic stress (Pu et al., 2017). We hypothesize that if the autophagy in the rns2-2 mutant is regulated via the TOR signaling pathway, then activation of TOR by auxin will result in the loss of autophagy activity.

To test this hypothesis, 7-day-old GFP-ATG8e and rns2-2 GFP-ATG8e expressing seedlings were transferred to half-strength MS liquid medium supplemented with 20 nM of auxin 1-naphthaleneacetic acid (NAA), for 6 hours, or DMSO as solvent control. After treatment, seedlings were visualized by confocal microscopy (Figure 2A) or pictures were taken using an epifluorescence microscope for quantification. In control conditions, GFP-ATG8e seedlings have a low basal level of autophagy while in rns2-2 GFP-ATG8e autophagy is active (Figure 2B). Activation of TOR kinase by NAA did not change the autophagy activity in GFP-ATG8e seedlings when compared to the solvent control (Figure 2B). In rns2-2 GFP-ATG8e seedlings, autophagy activity was blocked after activation of the TOR kinase by NAA treatment (Figure 2B). These results suggest that repression of the TOR signaling pathway will lead to the constitutive autophagy observed in the rns2-2 mutant.

To further confirm our results, we activated the TOR kinase through the brassinosteroid (BR) signaling pathway. Recent studies have shown a link between TOR signaling and BR signaling through the phosphorylation of TOR by the BR-regulated kinase Brassinazole-Insensitive 2 (BIN2) (Pu, 2017). Activation of BR signaling leads to inactivation of BIN2 (Kim et al., 2011; Nolan et al., 2017). Suppression of TOR phosphorylation by BIN2 leads to activation of TOR, therefore inhibiting autophagy. We hypothesize that if the constitutive autophagy in the rns2-2 mutant is regulated by TOR, then activation of TOR by activating the BR signaling pathway will result in the loss of autophagy activity.

To test this hypothesis, 7-day-old GFP-ATG8e- and rns2-2 GFP-ATG8e-expressing seedlings were transferred to half-strength MS liquid medium supplemented with 1 μM brassionolide (BL), the most active BR for 6 hours or DMSO as solvent control. After treatment, seedlings were visualized by confocal microscopy (Figure 3A) or pictures were taken using an epifluorescence microscope for quantification. In control conditions, GFP-ATG8e seedlings have a low basal level of autophagy while in rns2-2 GFP-ATG8e autophagy is active (Figure 3B). Activation of TOR kinase by BL did not changed the autophagy activity in GFP-ATG8e seedlings when compared to the solvent control (Figure 3B). In rns2-2 GFP-ATG8e seedlings, autophagy activity was blocked after activation of the TOR kinase by BL treatment (Figure 3B). Taken together, these results suggest that the autophagy in rns2-2 mutant is activated by the repression of the TOR signaling pathway, possibly by the suppression of TOR activity.

4.4 Discussion

Ribosome turnover is important for maintaining cellular homeostasis in response to nutrient deficiency and under normal conditions (Warner, 1999). RNS2, a vacuolar RNase, is necessary for rRNA decay in plants (Hillwig et al., 2011). In the absence of the RNase activity of RNS2, autophagy is activated as a compensatory mechanism to turn over the ribosomes (Floyd et al., 2015). This work focusses on understanding the mechanisms involved in the activation of autophagy in the *rns2-2* mutant. Our results suggest that suppression of the TOR signaling pathway is responsible for the activation of autophagy in *rns2-2* mutant.

In plants, a few regulators of the autophagy process are known, such as the ATG1 complex, which activates autophagy in response to nutrient starvation (Suttangkakul et al., 2011), IRE1b, which acts in response to ER stress (Liu et al., 2012), the TOR complex, a negative regulator of autophagy under starvation, salt and osmotic stress (Liu and Bassham, 2010; Pu et al., 2017), and SnRK1 complex, a positive regulator of autophagy in response to stress (Chen et al., 2017; Soto-Burgos and Bassham, 2017). The SnRK1 complex acts as an energy sensor, activating multiple signaling pathways in response to low energy levels (Baena-González et al., 2007). Inhibition of SnRK1 by T6P in the *rns2-2* mutant did not lead to changes in autophagy activity (Figure 1). Transcriptomic analysis of the *rns2-2* mutant indicated that these plants are not in a nutritional deficit status, and that 20% of the genes that are differentially expressed when compared to wild-type are targets of KIN10 (catalytic subunit of SnRK1) regulation (Morriss et al., 2017), suggesting that the SnRK1 pathway might be repressed in the *rns2-2* mutant.

The TOR complex regulates nutrient-responsive processes, such as growth, translation and autophagy (Anderson et al., 2005; Deprost et al., 2005; Liu and Bassham, 2010; Moreau et al., 2012). In nutrient rich conditions, the TOR kinase phosphorylates the ATG1 complex, leading to the suppression of autophagy activity (Kamada et al., 2010). Activation of the TOR kinase by auxin or brassinolide in the *rns2-2* mutant resulted in the inhibition of the autophagy activity (Figure 2 and 3). It is possible that in the *rns2-2* mutant, the TOR kinase activity is suppressed or reduced, leading to the constitutive autophagy phenotype. Further research is needed to address this hypothesis.

In conclusion, this study showed that the constitutive autophagy observed in plants without RNS2 activity can be repressed by the activation of the TOR signaling pathway. This suggest that the TOR signaling pathway is involved in the activation of constitutive autophagy in *rns2-2*, possibly by the suppression of TOR kinase activity. Further work is necessary to determine if other regulators of autophagy like the ATG1 complex are involved in the activation of autophagy in the *rns2-2* mutant. Another possibility to investigate is the mechanism by which elevated production of ROS (Morriss et al., 2017) works as a trigger for activating autophagy in *rns2-2* mutant. In *Chlamydomonas,* inactivation of ATG4 during oxidized conditions controls autophagy (Pérez-Pérez et al., 2016) and in mammals, ATG4 is redox regulated during starvation to promote autophagy (Scherz-Shouval et al., 2007). ATG4 function in the processing of ATG8 proteins for activation of autophagy and in the delipidation of ATG8-PE for ATG8 recycling (Yoshimoto et al., 2004). We, therefore hypothesize that ATG4 is inactivated by ROS, promoting lipidation of ATG8, potentially regulating the constitutive autophagy in *rns2-2*.

4.5 Materials and Methods

4.5.1 Plant materials and growth conditions

GFP-ATG8e (Xiong et al., 2007) and *rns2-2* GFP-ATG8e (Floyd et al., 2015) *Arabidopsis thaliana* seeds were surface-sterilized with 33% (v/v) bleach, 0.1% (v/v) Triton X-100 solution for 20 minutes, washed 5 times with sterile water, and kept at 4°C for at least 2 days in the dark. Plants were grown on half-strength MS medium (Murashige-Skoog with vitamins mixture [Caisson, MSP09], 2.4 mM MES [pH 5.7], 1% sucrose, 0.6% Phytoblend agar) or in soil under long day conditions (16 h light and 8 h dark) at 22°C.

4.5.2 Stress and chemical treatments

To inhibit SnRK1, 7-day old seedlings were transferred to half-strength MS liquid medium supplemented with 0.1 mM trehalose-6-phosphate (T6P) [Santa Cruz, SC216004] for 3 hours (Zhang et al., 2009).

To activate TOR, 7-day-old seedlings were transferred to half-strength MS liquid medium supplemented with 20 nM 1-naphthaleneacetic acid (NAA) [Sigma, N1641], 1 μM Brassinolide (BL) or DMSO as solvent control for 6 hours (Noguchi et al., 2000; Pu et al., 2017).

4.5.3 Microscopy

Seedlings were imaged for autophagosome quantification using a Zeiss AxioImager microscope, using a X40 objective and a fluorescein isothiocyanate (FITC) filter to visualize GFP fluorescence.

Confocal microscopy was performed using a Leica SP5 confocal laser scanning microscope with a X63 oil immersion objective. The excitation and emission wavelengths for GFP were 488 and 507 nm respectively. All of the microscopy was performed at the Roy J. Carver High Resolution Microcopy Facility at Iowa State University

4.5.4 Image and statistical analysis

Chemical treatments and microscopy were done blind and autophagosomes labeled with GFP-ATG8e in the elongation zone in the roots were manually counted. Only individual motile dots were counted as autophagosomes. A two-tailed student t-test was used to analyze the data and a p-value of 0.05 was used to determine significance.

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4.7 Figures

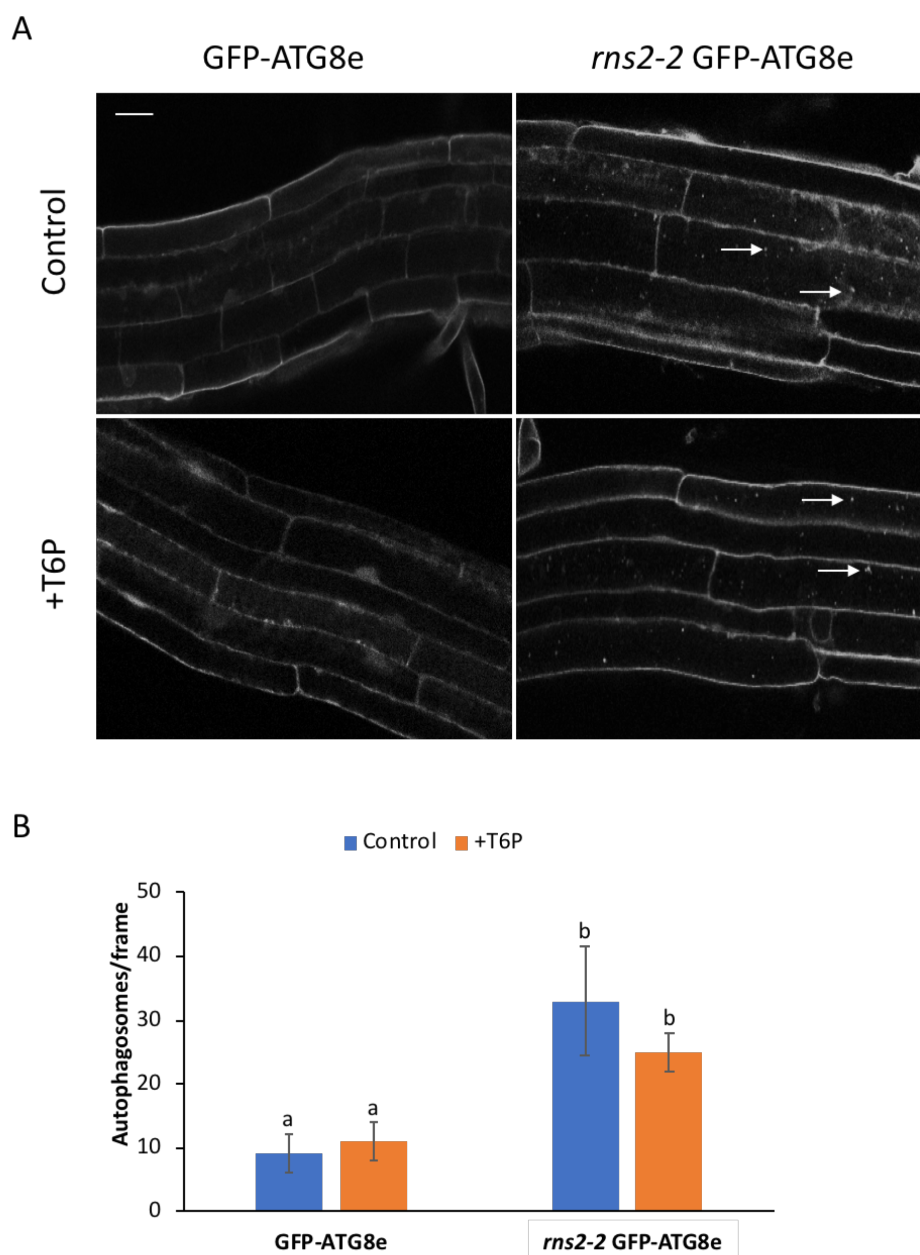

Figure 1. Trehalose-6-phosphate does not inhibit autophagy activity in *rns2-2* mutant

(A) GFP-ATG8e and *rns2-2* GFP-ATG8e seedlings were grown on ½ MS plates for 7 days. Seedlings were transferred to ½ MS liquid medium supplemented with 0.1 mM T6P for 3 hours. Confocal microscopy was used to visualize autophagosomes (white arrows) in roots. Scale bar = 25 μm. **(B)** Quantification of autophagosomes labeled with GFP-ATG8e in (A). Different letters denote statistical significance for three biological replicates with at least 9 frames per replicate, $p<0.05$, t-test. Error bars indicate standard error.

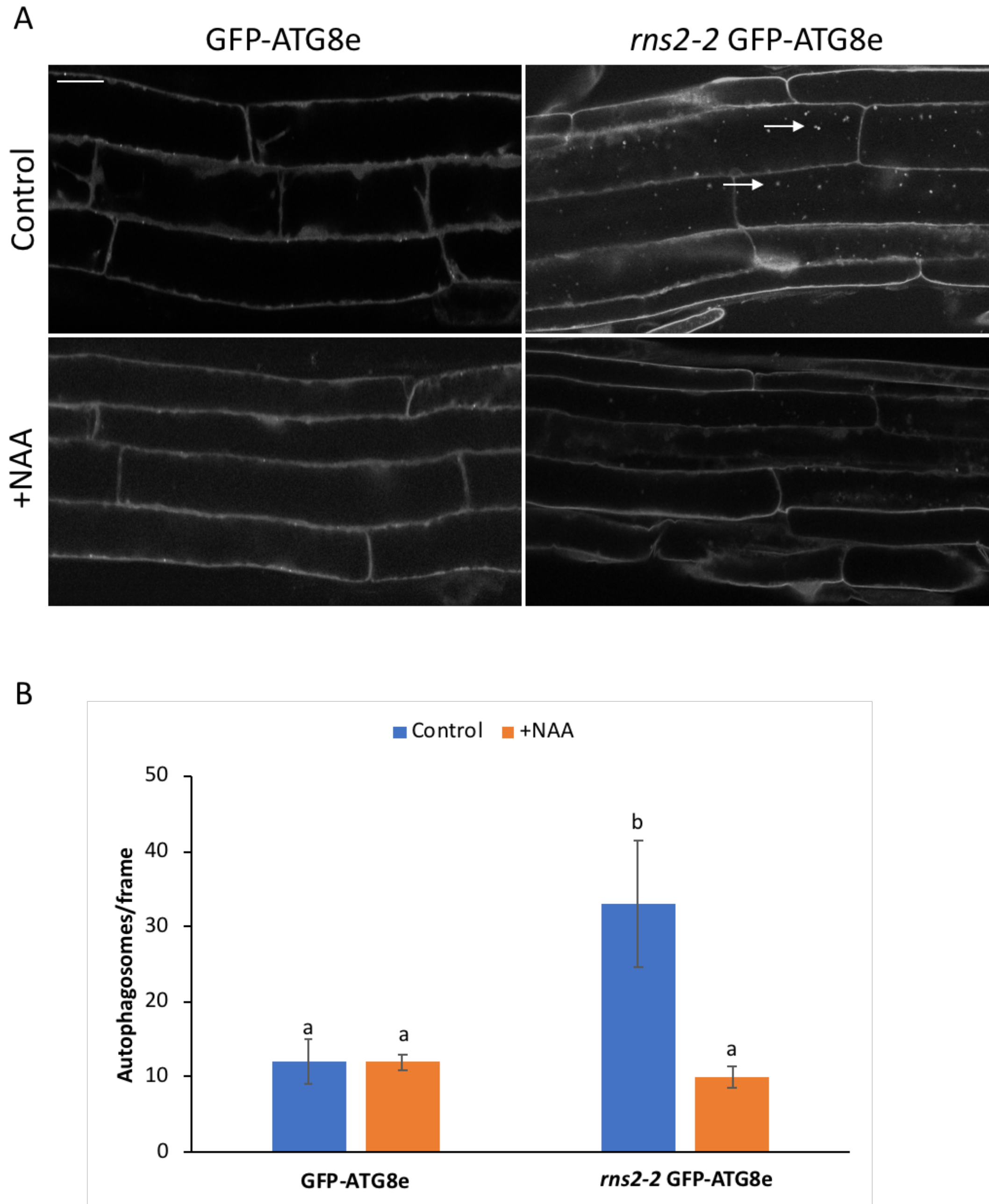

Figure 2. Auxin treatment inhibits autophagy activity in *rns2-2* mutant

(A) GFP-ATG8e and *rns2-2* GFP-ATG8e seedlings were grown on ½ MS plates for 7 days. Seedlings were transferred to ½ MS liquid medium supplemented with 20 mM NAA or DMSO for 6 hours. Confocal microscopy was used to visualize autophagosomes (white arrows) in roots. Scale bar = 25 µm. **(B)** Quantification of autophagosomes labeled with GFP-ATG8e in (A). Different letters denote statistical significance for three biological replicates with at least 9 frames per replicate, $p<0.05$, t-test. Error bars indicate standard error.

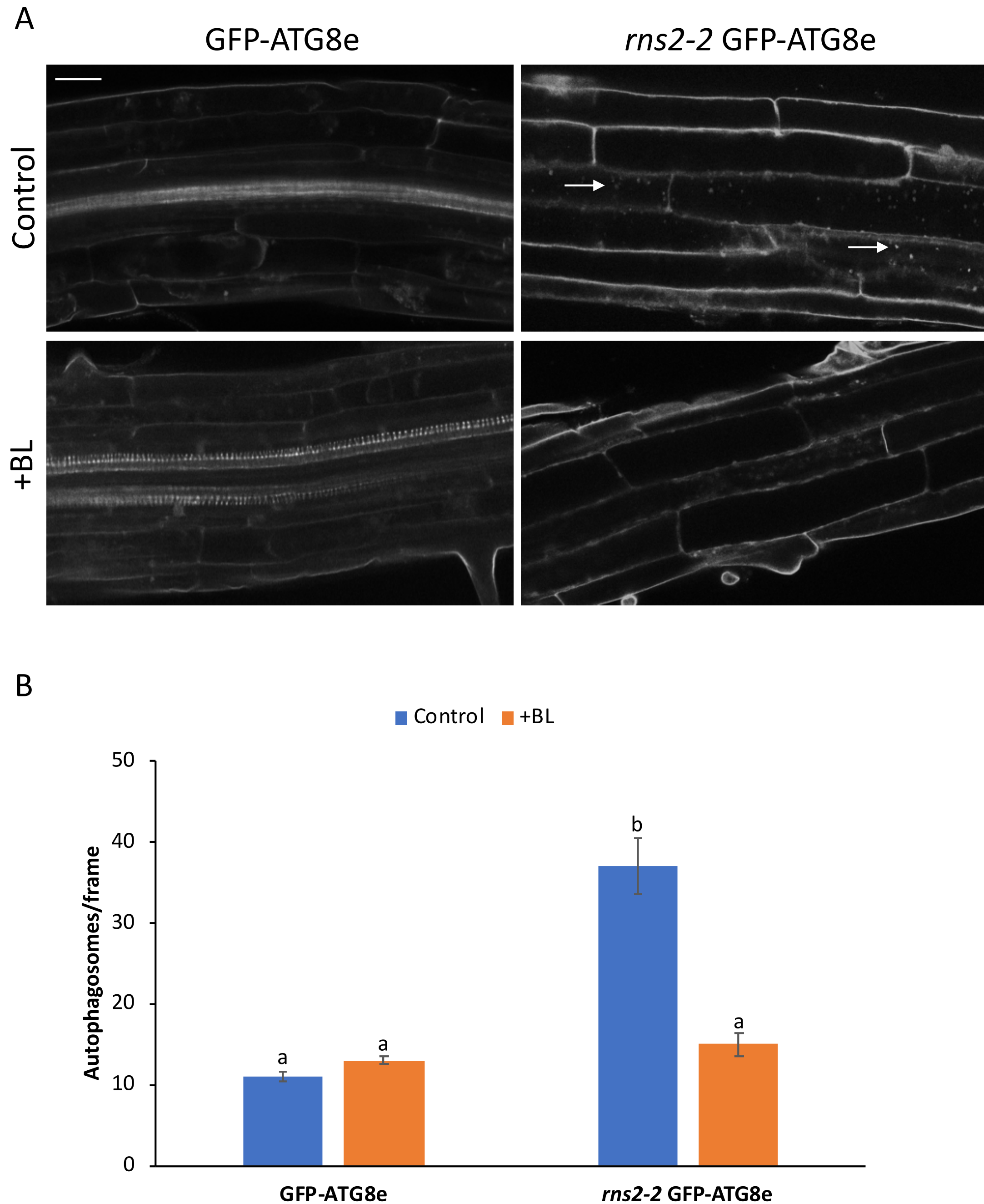

Figure 3. Activation of BR signaling inhibits autophagy activity in *rns2-2* mutant

(A) GFP-ATG8e and *rns2-2* GFP-ATG8e seedlings were grown on ½ MS plates for 7 days. Seedlings were transferred to ½ MS liquid medium supplemented with 1 μM BL or DMSO for 6 hours. Confocal microscopy was used to visualize autophagosomes (white arrows) in roots. Scale bar = 25 μm. **(B)** Quantification of autophagosomes labeled with GFP-ATG8e in (A). Different letters denote statistical significance for three biological replicates with at least 9 frames per replicate, $p<0.05$, t-test. Error bars indicate standard error.

CHAPTER 5. DISSERTATION DISCUSSION AND FUTURE WORK

5.1 Dissertation Discussion

This dissertation summarizes my work in investigating how is autophagy regulated in *Arabidopsis*. Three research questions were addressed: Is SnRK1 complex a regulator of autophagy and does it work through the TOR signaling pathway? Does the antioxidant molecule glutathione regulate autophagy during abiotic stress? What is the mechanism by which autophagy is activated in plants without RNS2 activity? Studies in these areas have furthered our knowledge and understanding about how autophagy is regulated in plants. I have shown that SnRK1 is a positive regulator of autophagy and works upstream of TOR or independently of TOR; glutathione is needed for autophagy activation under salt stress and nutrient starvation; and the TOR signaling pathway is involved in the activation of autophagy in *rns2-2* mutants.

5.1.1 SnRK1 regulates autophagy via TOR

Regulation of autophagy has been widely studied in animals and yeast but is still poorly understood in plants. A few regulators of autophagy have been identified in plants, such as the ATG1 complex, which positively regulates autophagy in response to nutrient stress (Suttangkakul et al., 2011), the TOR complex, a negative regulator of autophagy under nutrient rich conditions (Liu and Bassham, 2010) and IRE1b, which functions in response to ER stress (Liu et al., 2012). Most of the regulators identified in plants are downstream of the TOR complex, leaving a gap of knowledge for the regulators working upstream of TOR. This

dissertation advances our knowledge by identifying the SnRK1 complex as a positive regulator of autophagy upstream of TOR.

The SnRK1 complex is an energy sensor and during low energy conditions or metabolic stress it gets activated (Halford and Hey, 2009). Previous studies have demonstrated that the mammalian and yeast orthologs of SnRK1 can activate autophagy in response to low energy conditions by inactivation of the TOR complex (Wang et al., 2001). My studies demonstrate that the SnRK1 complex positively regulates autophagy. Overexpression of the catalytic subunit of SnRK1 complex, KIN10, or chemical activation of SnRK1 resulted in activation of autophagy. Activation of SnRK1 results in the upregulation of catabolism and downregulation of anabolism (Tsai and Gazzarrini, 2014), being autophagy, a potential mechanism used to maintain energy balance. In low energy conditions, autophagy recycles cytoplasmic components, producing both raw materials that can be used in biosynthetic pathways when substrates are limiting helping to maintain homeostasis.

Besides low energy, the SnRK1 complex has been linked other to plant stress responses including salt stress (Lovas et al., 2003) and pathogen resistance (Hao et al., 2003; Gissot et al., 2006). I have demonstrated that SnRK1 regulates autophagy in response to most abiotic stresses. Analysis of *kin10* mutant and chemical inhibition of the SnRK1 complex by T6P showed that KIN10 is necessary to activate autophagy during salt stress, oxidative stress, nutrient starvation and ER stress. During osmotic stress autophagy was only partially activated, suggesting that an alternative pathway may exist for activating autophagy under these conditions. A possibility is that additional, yet unidentified stress signals and/or sensors regulate autophagy upstream of TOR.

The TOR complex is a negative regulator of autophagy in many organisms (Noda and Ohsumi, 1998; Kamada et al., 2000; Liu and Bassham, 2010). In mammals, the SnRK1 ortholog, AMPK regulates autophagy via TOR signaling pathway or independently of TOR (Gwinn et al., 2008; Kim et al., 2011). Upon nutrient starvation, AMPK phosphorylates RAPTOR, resulting in the inhibition of TOR and leading to autophagy activation (Gwinn et al., 2008). My studies confirmed that KIN10 acts upstream of TOR in the regulation of autophagy in *Arabidopsis* via a TOR-dependent pathway. I show here that activation of autophagy by increasing KIN10 activity is blocked upon activation of TOR, whereas disruption of KIN10 activity does not block the constitutive autophagy seen upon inhibition of TOR. Recent studies have shown that KIN10 interacts with RAPTOR1B *in vivo* and can phosphorylate it *in vitro* (Nukarinen et al., 2016), suggesting the regulation of autophagy by SnRK1 in plants via TOR-dependent pathway works in a comparable manner as the mammalian AMPK.

Recent studies have shown that regulation of autophagy during ER stress or oxidative stress occurs via a TOR-independent pathway (Pu et al., 2017). During ER stress, autophagy activation is triggered by the accumulation of unfolded proteins (Yang et al., 2016) and requires the splicing factor IRE1b (Liu et al., 2012). AMPK/Snf1 can also regulate autophagy by direct phosphorylation of ATG1, leading to the activation of autophagy (Wang et al., 2001; Egan et al., 2011; Kim et al., 2011). Studies have demonstrated that KIN10 overexpression led to increased phosphorylation of ATG1 in response to sucrose starvation (Chen et al., 2017). My results showed that SnRK1 is required for autophagy activation during ER or oxidative stress, suggesting that, like AMPK, it can regulate autophagy through

a TOR-independent pathway, via ATG1 complex. Another possibility for ER stress autophagy regulation is that SnRK1 and IRE1b are in the same pathway, additional work is needed to test this possibility.

5.1.2 GSH regulates autophagy during salt stress and nutrient starvation

Studies in yeast and animals have suggested that glutathione (GSH) regulates autophagy in response to nutrient deficiency (Deffieu et al., 2009; Desideri et al., 2012), although this connection have not been made in plants to date. My studies in this dissertation demonstrate that GSH is required for autophagy activation during salt stress and nutrient starvation, advancing our knowledge in how autophagy is regulated in plants during these condtions.

GSH is an antioxidant that prevents damage to cellular components caused by ROS, by the scavenging of ROS (Aquilano et al., 2014). The production of ROS by the plasma membrane NADPH-oxidase is required for autophagy activity during salt stress and nutrient starvation (Liu et al., 2009). My studies showed that GSH is necessary for the activation of autophagy during salt stress and nutrient starvation. Autophagy induction by salt stress or sucrose starvation was blocked upon inhibition of GSH synthesis by BSO or in the glutathione-deficient mutant, *cad2-1*. Addition of exogenous GSH while inhibiting GSH synthesis with BSO during salt stress or sucrose starvation rescued the autophagy activation. Studies in animal cells, have shown that during starvation, scavenging of ROS is not the main mechanism by which GSH regulates autophagy (Desideri et al., 2012). Here, I demonstrate that quenching ROS using ascorbic acid in the presence or decrease of endogenous GSH resulted in autophagy inhibition upon salt stress. Addition of exogenous GSH partially activated autophagy in wild-type and *cad2-1* seedlings during salt stress plus

ascorbic acid. My results demonstrate that GSH, like in animals, works independently of ROS to activate autophagy.

Salt stress and nutrient starvation were the only abiotic stresses that required GSH to activate autophagy in response to these stresses. These stresses are regulated in a NADPH-oxidase dependent manner (Liu et al., 2009), which can act upstream of TOR (Liu and Bassham, 2010). My studies have demonstrated that regulation of autophagy by GSH acts upstream of TOR, via SnRK1 complex. Inhibition of GSH synthesis or adding exogenous GSH had no effect on the constitutive autophagy in the *raptor1b* mutant, while BSO treatment blocked the autophagy activity of KIN10 overexpression seedlings. Furthermore, addition of exogenous GSH while inhibiting GSH synthesis with BSO rescued the autophagy activity of KIN10 overexpression seedlings. It is probable that GSH regulates autophagy by activating the SnRK1 complex. I propose that upon salt stress and nutrient starvation, SnRk1 could be activated by GSH or by the ROS produced by NADPH-oxidase, leading to autophagy activation as a mechanism for maintaining cellular homeostasis.

5.1.3 TOR signaling pathway as a regulator of constitutive autophagy in *rns2-2*

Ribosome turnover is an important process for maintaining cellular homeostasis (Warner, 1999). The Arabidopsis T2 ribonuclease, RNS2, is necessary for rRNA decay and a loss of function mutation in *RNS2* leads to constitutive autophagy, as a compensatory mechanism for rRNA decay (Hillwig et al., 2011; Floyd et al., 2015). The studies presented in this dissertation show that in the *rns2-2* mutant autophagy activation occurs via the TOR signaling pathway, advancing our knowledge into the mechanisms by which autophagy is activated as a compensatory mechanism for rRNA decay.

The SnRK1 complex and TOR complex were candidates for regulating the constitutive autophagy in *rns2-2* mutant. I demonstrated that inhibition of SnRK1 complex in the *rns2-2* mutant did not lead to changes in autophagy activity. Studies in the *rns2-2* mutant revealed that *rns2-2* plants are not in a nutritional deficit status (Morriss et al., 2017), suggesting that the SnRK1 pathway might be repressed in the *rns2-2* mutant. In addition, I showed that activation of the TOR signaling pathway by auxin or brassinolide in the *rns2-2* mutant resulted in the inhibition of the autophagy activity. It is possible that in the *rns2-2* mutant, the TOR kinase activity is suppressed or reduced, leading to constitutive autophagy as a compensatory mechanism for rRNA decay.

In conclusion, the work presented in this dissertation has furthered our knowledge on how autophagy is regulated in plants in response to abiotic stress and by identifying regulators of autophagy working upstream of the TOR signaling pathway. This dissertation presents the first connection between GSH and autophagy in plants, opening a new area to be explored in relation to understand the mechanism of its regulation.

5.2 Impact of Dissertation

I have investigated the role of the SnRK1 complex and GSH in the activation of autophagy during abiotic stress. This has led to the identification of regulators of autophagy acting upstream of the TOR signaling pathway. The findings in this dissertation advances the plant autophagy field by providing a better understanding of how autophagy is regulated in response to abiotic stress. Understanding how autophagy is regulated in plants, can lead to the engineering of crops with better stress tolerance.

5.3 Future Work

5.3.1 Identification of upstream kinases that affect SnRK1 activity in response to stress

The SnRK1 protein kinase balances cellular energy levels in accordance with extracellular conditions and is thereby key for plant stress tolerance (Baena-González et al., 2007) In addition, SnRK1 has been implicated in many growth and developmental processes from seed filling and maturation to flowering and senescence (Tsai and Gazzarrini, 2014). Despite its importance, the upstream kinases that affect SnRK1 activity are poorly understood. To address this, a proteomics approach can be used to identify candidates that affect SnRK1 activity in response to stress. Recently, a new method has been described to address this type of questions named Fluorescence Complementation Mass Spectrometry (FCMS) (Zeng et al., 2017). This approach uses fluorescent protein fragments as originally developed for the bimolecular fluorescence complementation (BiFC) assay. For this study a fluorescent protein will be split into N-terminal and C-terminal fragments followed by fusion of the catalytic subunit of SnRK1, KIN10 with the N-terminal and the C-terminal will be fused to a library of kinases. The library of plant kinases will need to be generated. The vectors will be transiently expressed in protoplast followed by abiotic stress treatment. After treatment, the fluorescent protein will be immunoprecipitated to purify the protein complexes. Samples will then be prepared for analysis by liquid chromatography-mass spectrometry (LC-MS) to identify interacting kinases. This screen will provided candidates of upstream kinases in the SnRK1 signaling in response to abiotic stress.

5.3.2 Identification of the mechanisms of regulation of SnRK1

My studies demonstrated that SnRK1 complex and GSH can regulate of plant autophagy. I also showed, that GSH regulation of autophagy works upstream of TOR, possibly by activating SnRK1. However, how GSH control the autophagy via SnRK1 still need to be identified. In mammals, AMPK, SnRK1 ortholog, can be modified by S-glutathionylation in the presence of hydrogen peroxide and become active (Zmijewski et al., 2010). This possibility can be explored by immunoprecipitation of KIN10, followed by western blot analysis for S-glutathionylation modification using an anti-GSH antibody (Grek et al., 2012). To asses KIN10 activity, the specific substrate AMARA peptide can be used.

Recently, it was shown in *Arabidopsis* that SnRK1 is activated in reduced conditions and that its sensitivity is dependent of a conserved cysteine residue in the T-loop (Wurzinger et al., 2017). To determine if regulation of autophagy by GSH is dependent on redox status, transgenic plants with a single point mutation in the C200 residue can be created and autophagy assessed during stress conditions. These experiments will provide a better understanding of the mechanism that GSH uses to control SnRK1 activity and therefore, autophagy.

5.3.3 Characterization of regulatory mechanisms for autophagy activation in *rns2-2*

My studies showed that the constitutive autophagy observed in *rns2-2* can be repressed by the activation of the TOR signaling pathway. It is possible that suppression of TOR activity in *rns2-2* causes the constitutive autophagy observed. To address this hypothesis, TOR activity can be measure in *rns2-2* mutants. The phosphorylation of the TOR substrate, S6K, has been used as a marker of TOR kinase activity (Xiong et al., 2013; Li et al., 2017), therefore this method can be used to measure TOR activity.

In addition to TOR, there are other regulators of autophagy that could be involved in the activation of autophagy in the *rns2-2 mutant.* A potential candidate is the ATG1 kinase complex, which activity is regulated by nutritional status sensors such as TOR (Suttangkakul et al., 2011). To determine a function of the ATG1 complex in the activation of autophagy in *rns2-2,* a cross between *atg13a atg13b* double mutant and *rns2-2* mutant can be made to generate a triple mutant and assess the effect on the *rns2-2* constitutive autophagy phenotype.

Another candidate is the protease ATG4, which function in the processing of ATG8 proteins for activation of autophagy and in the delipidation of ATG8-PE (Yoshimoto et al., 2004). Inactivation of ATG4 during oxidized conditions controls autophagy in *Chlamydomonas* (Pérez-Pérez et al., 2016) and in mammals, ATG4 is redox regulated during starvation to promote autophagy (Scherz-Shouval et al., 2007). The mutant *rns2-2* has been shown to have elevated production of ROS (Morriss et al., 2017). I hypothesize that ROS works as a trigger for activating autophagy in *rns2-2* mutant, by inactivating ATG4. To address this hypothesis, a system to measure ATG4 activity described by (Woo et al., 2014) can be used. The first step is to prepare a construct of ATG8 tagged in the N-terminus with citrine fluorescent protein (C) and in the C-terminus with modified *Renilla* luciferase superhRLUC (ShR), C-ATG8-ShR. Creation of transgenic plants expressing C-ATG8-ShR in WT and *rns2-2* background can be used to asses ATG4 activity by western blot analysis using α-RLUC antibody. Completion of these experiments will provide a better understanding of the regulatory mechanisms for autophagy activation in *rns2-2.*

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