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Autophagy- and RNS2-mediated mechanism of ribosomal RNA degradation in Arabidopsis thaliana

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Autophagy- and RNS2-mediated mechanism of ribosomal RNA degradation in *Arabidopsis thaliana*

by

Stephanie Christine Morriss

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

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Program of Study Committee:
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  Drena Dobbs

Iowa State University
Ames, Iowa
2016

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DEDICATION

This dissertation is dedicated to my family, without whose love and support I would never have succeeded.

To my parents, Tina and Stephen Morriss, whose unwavering love and unconditional support has allowed me to venture outside of my comfort zone.

To my cousin, Ryan, whose child-like joy and enthusiasm is a constant source of rejuvenation.

To Grandpa, who has always been a source of quiet support.

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Ribosomal RNA degradation is necessary to remove nonfunctional rRNA and maintain homeostasis through degradation of aging or normal rRNA. While pathways for surveillance of nonfunctional rRNA have been characterized, mechanisms of degradation of normal rRNA are not yet well understood. Ribosomal RNA, the majority of cellular RNA, forms a massive resource sink. These studies examine the mechanism of degradation of rRNA in Arabidopsis and the effects of disruption of this process through mutation of key genes.

Ribosomal RNA in many organisms, including yeast, zebrafish, Tetrahymena and Arabidopsis, is degraded by the RNase T2 family of enzymes, a family highly conserved throughout eukaryotes. Mutation of the Arabidopsis RNase T2, RNS2, results in increased rRNA half-life and constitutive autophagy. An autophagy-dependent mechanism of ribosomal degradation, known as ribophagy, has been shown in yeast but not plants. Here, I report the effects of mutation of autophagy genes on rRNA accumulation in Arabidopsis. I show that RNA accumulates in rns2-2 and autophagy mutants atg9-4 and atg5-1, indicating that RNS2 and the autophagy machinery are necessary to maintain normal rRNA levels in Arabidopsis. This accumulation is vacuolar in rns2-2 but not atg5-1. These evidence support an autophagy-dependent rRNA degradation by RNase T2 in plants.

To further examine the effects of mutation in this rRNA degradation pathway, I performed two transcriptomic studies. My initial analysis of WT and rns2-2 in seedlings revealed differentially expressed genes (DEG) related to carbon flux pathways and cell wall modifying processes. Alteration of carbon flux in the mutant was verified using metabolite
analysis, while the impact on cell wall was studied using cell wall component analysis and phenotypic data which revealed that rns2-2 plants are larger due to an increase in water content and cell elongation. I next performed an expanded transcriptomic analysis including WT, rns2-2, atg9-4, and rns2-2atg9-4 in adult plants and found cell wall and oxidative stress associated DEG. Further analysis indicated a role for reactive oxygen species signaling constitutive autophagy in rns2-2 plants. These results support existence of a ribophagy-like mechanism in Arabidopsis and dissect the importance of rRNA degradation in maintenance of cellular homeostasis in plants.
CHAPTER I
INTRODUCTION: RIBOSOMAL RNA DEGRADATION

1.1 Ribosomal degradation

Several mechanisms are necessary for the degradation of ribosomal RNA. Ribosome assembly is a complicated process including many components, such as the ribosomal RNA, ribosomal proteins, and around 140 additional proteins [1, 2]. Ribosomal RNA undergoes several posttranscriptional modifications and, at each stage in maturation, must be tested for quality.

The synthesis of ribosomes is a multistep process (reviewed: [3, 4]) which requires each step to be completed successfully. Persistent misassembled ribosomes could result in significant wastage of cellular resources and energy as well as cause a high risk of damage to the cell through non-functioning or mis-functioning proteins with potentially devastating effect [5, 6]. The systems that ensure the functionality of ribosomal RNA are called surveillance pathways and are the best described pathways for ribosomal RNA decay. Aberrant 5S rRNA is retained in mutants in the enzyme responsible for polyadenylating misprocessed tRNA, Trf4p [7], illustrating the role of polyadenylation in rRNA surveillance [8, 9]. In yeast, polyadenylation of ribosomal RNA has been identified as a quality control mechanism in rRNA processing during synthesis [10]. The TRAMP (Trf4p/Air/Mtr4p polyadenylation) complex, is a polyadenylation complex linked both to pre-rRNA processing and degradation of aberrant rRNA [11]. Defective rRNAs are degraded by the exosome, a complex of proteins with a 3’-5’ exonuclease activity [12].

Mature nonfunctioning ribosomal RNA has its own pathway for degradation, nonfunctioning rRNA decay (NRD) [13]. NRD was identified through point mutations in the
yeast 18S and 25S ribosomal RNA which specifically allow full processing of the ribosomal RNA to yield mature yet ineffective ribosomes [13]. These mutations resulted in decreased stability in the ribosomal RNA, as seen in northern blot analysis and a transcriptional shut off analysis, although the synthesis of these mutant rRNA were not impaired as demonstrated by pulse chase experiments [13]. The protein NMD3 is necessary for 25S rRNA stability. Mutants in NMD3 (nonsense-mediated decay 3) have precursor 25S rRNA kinetics similar to WT but decreased stability of the mature rRNA, indicating a role for NMD3 in rRNA surveillance [14].

Ubiquitination also serves as a signal in nonfunctional ribosomal RNA decay. Knockout of proteins in yeast have revealed that two components of an E3 ubiquitin ligase complex, Mms1 and Rtt101, are required in the 25S NRD pathway [15]. Additionally, it was found that, in yeast expressing nonfunctional 25S rRNA, there is an enrichment of ubiquitinated proteins in ribosomal fractions [15]. A mechanism for degradation of ribosomal RNA through the autophagy pathway has been proposed.

1.2 Autophagy

During macroautophagy cargo, from individual proteins to full cellular organelles, is encapsulated by double-membrane vesicles called autophagosomes, which convey the cargo to the vacuole for degradation by hydrolases [16, 17]. Autophagy is performed by a number of autophagy-associated genes known as ATG genes. Autophagy in yeast requires two ubiquitin-like conjugation systems, the Atg8 and Atg12, and plants require similar systems with multiple members of the proteins from this family [18]. Atg8 undergoes processing by the cysteine protease Atg4, exposing a glycine to the E1-like Atg7 [19-22]. Atg8 is then transferred to the E2-like Atg3 and is conjugates to phosphatidylethanolamine [23]. Similarly, Atg12 is activated by
Atg7 and transferred to Atg10, another E2-like enzyme, after which it is conjugated to Atg5 and interacts with Atg16 [24, 25]. Both of these ubiquitin-like systems reside in the preautophagosomal structure. Additionally, the preautophagosomal structure requires a PtdIns3K complex, potentially to recruit PtdIns(3)P binding proteins such as Atg18 to the preautophagosomal structure [26].

This process next involves ATG9, a membrane-bound ATG gene containing six to eight transmembrane domains as predicted by hydrophobicity analysis, which fluorescent localization studies reveal is involved with the preautophagosomal body, the phagophore [27, 28]. During the phagophore formation, as observed in high temporal-resolution microscopy, ATG9 recruits lipids to form the double membrane via ATG9-containing vesicles which originate from the Golgi [29]. Loss of phagophore localization in ATG17 deficient cells indicates that ATG9 is recruited by ATG17 to the phagophore [30]. Immunoprecipitation shows that ATG17 and ATG9 interact and that the equilibrium interaction between these two proteins is dependent on the protein kinase activity of ATG1, but ATG1 is not required for localization to the phagophore [30]. Indeed, in already formed mouse autophagosomes, mutation of ATG9 reveal no disruption of integration of further downstream components of autophagy, supporting that ATG9 is responsible for vesicle formation but is not an integral component of the mature autophagosome [31]. ATG9 is retrieved from the autophagosome and does not become a component of the vacuolar membrane in spite of being in the outer autophagosomal membrane [29], with retrieval of ATG9 being dependent on the ATG1-13 complex, ATG2, ATG18 and phosphatidly-inositol-3-phosphate generated by an ATG14-containing complex [32]. Any mutation of these components results in dysfunctional autophagosomes [32]. As demonstrated in ATG9 deficient yeast, without ATG9 the autophagosome will not form [28]. After formation, the autophagosome is delivered to the
vacuole for degradation. Delivery to the vacuole involve several SNARE proteins for fusion [33].

There exist a number of selective autophagy processes, many of which have been identified first in yeast. Some forms of selective autophagy are part of the biosynthetic processing pathway for vacuolar or lysosomal proteins such as the cytoplasm-to-vacuole targeting pathway which transports certain hydrolases to the vacuole [34]. Selective autophagy is also used to maintain homeostasis [35] and perform quality control for damaged or superfluous organelles, such as in mitophagy, the selective autophagy of mitochondria [36-40], pexophagy, the selective autophagy of peroxisomes [41-43], PMN, piecemeal microautophagy of the nucleus [44-46], and reticulophagy, selective autophagy of the endoplasmic reticulum [37, 47-49]. Ribosomes may be captured alongside endoplasmic reticulum due to their association with the rough endoplasmic reticulum. They also have their own forms of selective autophagy, including the selective macroautophagy of ribosomes, called ribophagy [50]. Additionally, in animals, a transporter-mediated mechanism of ribosomal RNA uptake into the lysosome exists known as RNautophagy [51].

Selective autophagy appears to have a few common principles. The role of selective autophagy is maintaining homeostasis, either through degradation of damaged or misfolded structures, or through response to environmental or cellular conditions resulting in either a need for changes in level of that organelle or structure, or recycling of the components of that structure for repurposing [35]. There appear to be two core pathways for recognition of the cargo. One of these mechanisms includes factors that specifically bind to the cargo and act as signals for vacuolar transport to the vacuole, potentially serving as an anchor point for the formation of the double membraned vesicles known as autophagosomes [16]. A second
mechanism includes ubiquitin acting as a signaling molecule \[52, 53\]. Once the ubiquitin is bound to the cargo, an adaptor can then signal it for inclusion into the autophagosome \[53\].

Autophagy is induced by a great number of stimuli, including starvation and oxidative stress. Soluble sugars, including sucrose, glucose and fructose, can all signal oxidative stress. Metabolic reactions and regulations link soluble sugars with the production of reactive oxygen species, including through respiration and photosynthesis as well as oxidative pentose-phosphate processes and carotenoid biosynthesis \[54\].

Autophagy can be involved in senescence and stress response. In animals, autophagy is a lysosomal degradation pathway that serves in cellular responses and tumor suppression \[55\]. Autophagy enables adaptation to stress by degradation of cellular components. Autophagy maintains mitochondrial metabolism. In cells expressing an active form of Ras, autophagy was constitutive and showed a limited ability to be induced by starvation. These cells were analyzed by LC-MS for TCA cycle component pools (specific to the mitochondria and results showed that this metabolites were lower in the Ras-expressing (defective autophagy) cells which were either homozygous in WT \textit{ATG5} (atg5 +/+ ) or homozygous in mutant \textit{ATG5} (atg5 -/- )-cells. Also, Pyruvate was higher in atg5 -/- cells. These autophagy defective cells also show a reduced oxygen potential as measured through oxygen consumption rate. The cells also had a greater response to starvation impact on Energy charge, with a large drop in autophagy defective cells \[56\].
1.3 Ribophagy

Ribophagy is the selective autophagy of ribosomes, during which ribosomes are taken by autophagosomes to the vacuole for degradation. It was initially identified in yeast, however ribophagy-like mechanisms have been identified in other organisms, including Arabidopsis. In yeast, nitrogen starvation induces accumulation of fluorescent RPL25 and RPL5 as well as RPS2P and RPS3P in the vacuole [50]. This accumulation was dependent on the autophagic machinery as well as the cytoplasm-to-vacuole targeting pathway component Atg19 [50]. Indeed, when the levels of free GFP were measured in these yeast, it was found that cleaved GFP accumulated in WT cells overexpressing the RPL25-GFP fusion but not in ATG7 mutants [50]. This autophagy-dependent degradation of ribosomes is dependent on the ubiquitin-specific protease Ubp3 and the Ubp3 co-factor Bre5, neither of which cause general autophagy defects, indicating the need to cleave a ubiquitin signal from an unknown protein(s), possibly the ribosomal proteins, to signal the selective autophagy of the ribosome [50]. During ribophagy in yeast 60S ribosomal subunit targeting is dependent on Ubp3 and Rsp5, an associated E3 ubiquitin ligase. Genetic screening in yeast revealed that while the rsp5 mutation alone did not cause a defect in ribophagy, combination of the rsp5 and ubp3 mutations causes a stronger disruption than mutation of Ubp3 alone [57]. Similarly, the chaperone-like protein Cdc48 and one of its ubiquitin binding adaptor Ufd3 were identified in this complex required for ribophagy through immunoprecipitation assays using tagged Bre5 or Ubp3 [58].
1.4 RNase T2

RNases belonging to the RNase T2 family are found throughout Eukaryotes [59-63]. These enzymes are characterized by a conserved sequence around the active site. Although the RNase T2 family was originally characterized to contain acid ribonucleases [64], neutral ribonuclease members have since been discovered [63], indicating the RNase T2 family is not limited to acid ribonucleases. RNases T2 are traditionally viewed to be base non-specific and act upon single stranded RNA. Some RNases T2 have shown preferences for cleaving at certain bases, such as the guanidine-preferring RNase NW from *Nicotiana glutinosa* [65] and the uridine-specific MC1 from bitter gourd [66, 67]. However, many others have a broad-specificity including *Nicotiana glutinosa* RNase NT [68], squid RNase Tp [69] and RNase LE from *Lycopersicon peruvianum* [70]. RNA endonucleolytic cleavage occurs through a 2-stage process with a cyclic 2’,3’-phosphate intermediate [71]. RNases T2 are either retained in the vacuole or secreted from the plant cell [60]. RNase T2 are often glycosylated [62, 63, 71-74]. In yeast, this glycosylation has been linked to subcellular residency as the yeast RNase T2 enzyme, Rny1p, can be either secreted or retained in the vacuole based on glycosylation [75]. However, no evidence exists to support this mechanism in plants where glycosylation more likely assists in protein folding and stability [76, 77]. Structural analyses for several RNases T2 have been completed, including RNase NW (protein product of the *NGR3* gene) in *Nicotiana glutinosa* [65], RNase NT (protein product of the *NGR1* gene) in *Nicotiana glutinosa* [68], RNase MC1 from bitter gourd [66], RNase Rh from *Rhizopus niveus* [71], the S3-RNase from *Pyrus pyrifolia* [78], S3-RNase from *Lycopersicon peruvianum* [79], RNase LE from *Lycopersicon peruvianum* [70] and the human RNaseT2 [80]. These structures reveal interactions around the active site which include three histidines as well as, depending on the ribonuclease, coordinating
tryptophans, aspartates, asparagines, glutamine and lysine and structure-maintaining disulfide bonds [65, 68, 71, 74, 79].

While in animals there is primarily one RNase T2 per species, in plants this family has undergone a broad diversification into three classes of ribonuclease known as Classes I-III [81]. The Class I RNases T2 are diverse and regulated by diverse stimuli, including wounding and pathogen response, as reviewed by MacIntosh [82]. Some of the stimuli regulating their expression include the induction of *Nicotiana glutinosa* RNase NW in response to tomato mosaic virus infection [83], induction of *Nicotiana tobacum* RNase NE during *Phytophthora parasitica* attack [84], and induction of Arabidopsis RNS1 in response to wounding [85]. This class has undergone gene duplication or inactivation producing the most diversity among the RNase T2 enzymes in plants and, in phylogenetic analyses of the plant RNase T2 family, the clade containing the Class I enzymes is less well-structured than that of the highly conserved Class II enzymes [60, 86]. In addition to this highly structured phylogeny, the presence of Class II enzymes is highly conserved across plants. The Class II enzymes have the highest homology to the animal RNase T2 [60], are constitutively expressed [87] and have been linked to degradation of ribosomal RNA [63]. In contrast to the ubiquitous Class II enzymes, Class III RNase T2 enzymes, also known as S-RNases, are a highly specialized class of RNases T2 found in only three plant families, Solanaceae, Scrophulariaceae, and Rosaceae [81], and are responsible for self-incompatibility in pollination [88]. Studies of this self-incompatibility mechanism have progressed furthest in Solanaceae and has been completed in *Petunia inflata* [89]. The S-RNase in the pistil determines compatibility through interaction with the S-locus F-box in the pollen [88].
Whereas Class II are primarily vacuolar, as seen in Class II RNase T2 in rice [60] and RNS2 from Arabidopsis [63, 90], Class I and Class III are predominantly extracellular, as in RNS1 from Arabidopsis [90] and self-incompatibility in Nicotiana alata [91, 92]. Class II enzymes are arguably the most important as their ubiquitous presence and high conservation indicates a conserved role which was established to be ribosomal RNA degradation [63].

Strong support exists for a role for RNases T2 in ribosomal RNA degradation. Indirect evident for the role of RNase T2 in RNA homeostasis includes the regulation of RNase T2 in response to phosphate starvation. Northern blots against RNase LE from Nicotiana alata reveal RNase LE is induced in response to phosphate starvation [93]. Additionally, RNS2 in Arabidopsis is regulated by phosphate starvation [94]. Similar induction by phosphate starvation is shown in Antirrhinum AhSL28 [95]. Current hypotheses suggest that RNase T2 enzymes are part of a scavenging mechanism that recovers phosphate from RNA during times of scarcity [96].

RNase T2 in zebrafish and humans degrades ribosomal RNA. The RNase T2 in zebrafish, RNASET2, localizes to the lytic body of the animal cell, the lysosome, as identified in immunofluorescent staining in HEK293 cells expressing zebrafish RNASET2. The zebrafish genome contains two RNase T2 genes, a duplication unique to fish [59]. In a zebrafish RNase T2 null mutant, rnaset2, staining with acridine orange reveals enlarged lysosomes and in situ hybridization for the 28S rRNA revealed ribosomal RNA accumulation in the lysosome. Further, in these mutant zebrafish, white matter lesions containing RNA form which resemble the lesions formed in humans suffering from familial cystic leukoencephalopathy [97]. Patients with this disease have mutation in the human RNase T2, RNASET2, which may result in multiple effects,
such as mislocalization to the ER and misfolding of the protein [98]. These results indicate a strong association between RNase T2 in animals and ribosomal RNA degradation.

The relationship between RNase T2 and ribosomal RNA degradation is upheld in the ciliate *Tetrahymena thermophila*, which contains eight RNase T2 genes, known as *RNT2A-RNT2H* [61]. Mutation of a single *RNT2* gene resulted in no or minimal impact indicating functional redundancy, however upon mutation of multiple *RNT2* genes accumulation of ribosomal RNA fragments is observed in starved cells, showing an RNase T2-dependent, starvation-induced turnover of ribosomal RNA in tetrahymena [61].

In yeast the RNase T2, Rny1, is responsible for ribosomal RNA degradation. Yeast cells deficient in Rny1 were larger and had less uniformity than WT [73]. *RNY1* expression is regulated by a number of stresses, including heat shock, osmotic stress and oxidative stress [73, 99]. Under oxidative stress in yeast, ribosomal RNA is fragmented, as seen in northern blots probing the 25S rRNA [99]. Ribosomal RNA accumulates in cells carrying *rny1* mutations [99, 100].

### 1.5 RNS2

Arabidopsis has five annotated RNase T2 genes. Only one of these genes, *RNS2*, is found throughout the plant [101], is expressed constitutively at a high level, and is annotated as an RNase T2 Class II. The high level of expression of *RNS2* was determined initially by Northern blots and later confirmed through protein and transcriptome analyses. Western blotting against RNS1 and RNS2 from protein extracted from Arabidopsis and Northern blotting against *RNS2* mRNA revealed RNS1 and RNS2 are induced by phosphate starvation [90, 94, 101]. A plant deficient in phosphate delivery into the xylem, *pho1*, which has lower phosphate in the leaves,
has induction of \textit{RNS2} and \textit{RNS1} [94]. \textit{RNS2} is expressed at higher levels during senescence [94, 101]. This strong correlation between expression and phosphate limitation supports involvement of either \textit{RNS1} or \textit{RNS2} with ribosomal RNA degradation, a process by which the cell can recover phosphate for reuse in the cell. However, Western blotting reveals \textit{RNS1} is extracellular while \textit{RNS2} is intracellular, supporting a role for \textit{RNS2} in ribosomal RNA degradation [90]. Further, \textit{RNS2} has a C-terminal extension which most likely acts as a vacuolar localization signal [90]. Indeed, colocalization of YFP-HDEL with \textit{RNS2}-CFP as well as a diffuse vacuolar signal show \textit{RNS2} is expressed in the ER and vacuole, as seen in both transiently transformed protoplasts and transgenic roots [63]. Treatment with N-glycosidase F revealed that the multiple \textit{RNS2} bands present in RNase activity zymograms are caused by glycosylation of \textit{RNS2} [63]. A subcellular fractionation method dependent on ultracentrifugation reveals \textit{RNS2} activity is enriched in ER-associated fractions, as well as in the soluble cytosolic/vacuolar fraction [63]. Subsequent RNase activity assays on purified vacuoles reveal \textit{RNS2} activity is high in the vacuoles [63]. \textit{RNS2} expressed heterologously in yeast is most active at a pH of 7.5 while \textit{RNS2} from plants is highly active at a neutral pH, yet still maintaining significant activity at more acidic pH, including pH 5.0, the approximate pH of the vacuole [63]. This could reflect tuning of activity by pH or the necessity of \textit{RNS2} to be active in more than one location, such as in the ER, which has a neutral pH. \textit{RNS2} activity is not dependent on zinc and is inhibited by zinc levels above 2 µM [102].

The role of \textit{RNS2} in ribosomal RNA degradation in Arabidopsis was first reported by Hillwig et al. (2011). In this study, WT and \textit{rns2-2} roots stained with SYTO-RNASelect, an RNA specific fluorescent dye, exhibit significantly higher fluorescence in the \textit{rns2-2} stained roots than in the WT [63]. Staining appeared to be primarily in the vacuole, although some
diffuse cytoplasmic staining around the periphery of the vacuole appeared in a subset of samples [63]. Protoplasts from WT and rns2-2 plants revealed that, in addition to the vacuolar staining, a minority of rns2-2 cells contained stained puncta within the vacuole which were not observed in the WT [63]. To determine whether the increase in RNA was due to rRNA and ascertain the involvement of RNS2 in ribosomal RNA degradation, rRNA half-life was calculated using a pulse-chase experiment with radiolabeled H\textsuperscript{3}-Uridine. This method revealed that the ribosomal RNA in rns2-2 has a significantly longer half-life than that in WT [63].

The constitutive autophagy phenotype of rns2-2, combined with the vacuolar localization of the RNS2 protein [63] are consistent with the involvement of RNases T2 in an autophagy-dependent degradation mechanism for rRNA. This autophagy-dependent mechanism resembles yeast rRNA degradation, wherein Rny1p in the vacuole degrades rRNA through an autophagy-dependent selective mechanism [100], although this degradation of rRNA in yeast is starvation-dependent [58] while in plants it is a housekeeping function not dependent on starvation [63].

1.6 Objectives

This evidence for a ribophagy-like mechanism in plants leads us to two important questions: What is the mechanism for rRNA degradation in Arabidopsis? Why is there a constitutive autophagy phenotype in rns2-2? My research examines these two questions. Our first objective was to identify whether the ribophagy-like mechanism existed in plants. The second objective was to identify the impact of mutation in the ribophagy-like mechanism, to look for any signal which could trigger the constitutive autophagy phenotype and examine the impact of disruption of ribosomal RNA decay.
1.7 Dissertation Organization

This dissertation covers my work regarding the degradation of ribosomal RNA in Arabidopsis by the RNase T2 family in an autophagy-dependent mechanism and the effects of mutation of the ribosomal RNA degradation machinery including RNS2 and ATG9.

Chapter 1 serves as a general introduction to the topic of ribosomal RNA degradation, autophagy and the RNase T2 family of ribonucleases and was drafted by me.

Chapter 2 explains the relationship between RNS2 and autophagy. We find that both RNS2 and the autophagy components ATG5 and ATG9 are required for ribosomal RNA degradation and homeostasis. Autophagy mutants and double mutants between autophagy and RNS2 are much smaller than WT, and RNS2 induces constitutive autophagy wherein RNA is present in the autophagosomes. RNA accumulates in rns2-2, atg9-4, atg5-1, rns2-2atg9-4, and rns2-2atg5-1, highlighting the importance of RNase T2 and autophagy in ribosomal RNA degradation and plant cell homeostasis. This accumulation is vacuolar in rns2-2 but not in the autophagy mutants, except for rns2-2atg9-4 which may have activated an alternative method of import of RNA into the vacuole which is not dependent on ATG9. I generated the rns2-2 ATG8-GFP used to verify the presence of autophagosomes. Brice Floyd generated the rns2-2atg9-4 and rns2-2atg5-1 double mutants. Brice Floyd performed all microscopy work. I performed the RNase activity gel analysis and quantification of total RNA. Extraction of vacuoles was a joint activity between Brice Floyd and myself. Brice Floyd performed the qPCR analysis after vacuole extraction and I performed the acid phosphatase measurement and statistical analysis. Division of written components of the article followed the division of labor in the experiments. This chapter is published [Floyd, Brice E., et al. "Evidence for autophagy-dependent pathways of rRNA turnover in Arabidopsis." Autophagy 11.12 (2015): 2199-2212].
Chapter 3 develops the impact of loss of function of RNS2 in seedling homeostasis. We find that mutation of RNS2 results in minor transcriptomic changes, in contrast to the disruption to homeostasis we see through constitutive autophagy and RNA accumulation, supporting the existence of posttranscriptional or posttranslational regulation of the response to RNS2 loss. We find two classes of overrepresented differentially expressed genes involve the cell wall and the carbon flux pathways. We verified our cell wall results through phenotypic analyses and quantification of cell wall components. RNS2 mutation results in larger plants, longer root lengths, longer root cell lengths and increased water content, potentially as an effect of weaker cell walls and increased osmolyte content through RNA accumulation. The cell wall components mannose and glucuronic acid are lower in rns2-2 plants. We also verified our carbon flux relationship through metabolite analysis. We found that non-specific analysis revealed lower levels of glucose, fructose and sedoheptulose in rns2-2 plants and pentose phosphate pathway specific analysis revealed lower levels of sedoheptulose-7-phosphate as well as an unresolved peak representing ribose-5-phosphate, ribulose-5-phosphate and xylulose-5-phosphate in rns2-2 plants. Xiaoyi Liu performed the metabolite measurements and Brice Floyd contributed the rosette measurements. I completed all other experiments and analysis, with technical assistance from Andrew Severin on use of affylm for differential gene analysis in microarray. This chapter has been submitted for publication.

Chapter 4 presents the results of expanded transcriptomic analysis of rns2-2 and additionally atg9-4 and rns2-2atg9-4 in adult plants. We again find that the transcriptomic effects of loss of RNS2 are minor compared to the impact on the plant homeostasis, with few differentially expressed genes. Results support the importance of cell wall modifying proteins in response to loss of normal ribosomal RNA degradation in the adult plants, represented in all
three genotypes. Further, a novel relationship was discovered between disruption of ribosomal RNA degradation and oxidative stress, which was verified through staining for reactive oxygen species which finds rns2-2 is in a high oxidative state, as well as inhibition of reactive oxygen species production by NADPH oxidase that caused the loss of the constitutive autophagy phenotype, potentially revealing the signal that controls constitutive autophagy in rns2-2 plants. Xiaoyi Liu provided metabolite measurements and Brice Floyd imaged and measured the oxidative stress experiments. I performed all other experiments and analyses, with technical assistance from Michelle Graham for bioinformatics for RNA-seq analysis.

Chapter 5 integrates the results of the preceding chapters and provides a synthesis of information available regarding ribosomal RNA degradation through the autophagy-dependent pathway by RNase T2, evidence of a homeostatic ribophagy-like process in plants.
1.8 References


CHAPTER 2
EVIDENCE FOR AUTOPHAGY-DEPENDENT PATHWAYS OF RRNA TURNOVER IN ARABIDOPSIS

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2.1 Abstract

Ribosomes account for a majority of the cell’s RNA and much of its protein and represent a significant investment of cellular resources. The turnover and degradation of ribosomes has been proposed to serve a role in homeostasis and during stress conditions. Mechanisms for the turnover of ribosomal RNA (rRNA) and ribosomal proteins have not been fully elucidated. We show here that the RNS2 ribonuclease and autophagy participate in RNA turnover in *Arabidopsis thaliana* under normal growth conditions. An increase in autophagosome
formation was seen in an *rns2*-2 mutant, and this increase was dependent on the core autophagy genes *ATG9* and *ATG5*. Autophagosomes and autophagic bodies in *rns2*-2 mutants contain RNA and ribosomes, suggesting that autophagy is activated as an attempt to compensate for loss of rRNA degradation. Total RNA accumulates in *rns2*-2, *atg9*-4, *atg5*-1, *rns2-atg9*-4, and *rns2-2atg5*-1 mutants, suggesting a parallel role for autophagy and RNS2 in RNA turnover. rRNA accumulates in the vacuole in *rns2*-2 mutants. Vacuolar accumulation of rRNA was blocked by disrupting autophagy via an *rns2-atg5*-1 double mutant but not by an *rns2-atg9*-4 double mutant, indicating that *ATG5* and *ATG9* function differently in this process. Our results suggest that autophagy and RNS2 are both involved in homeostatic degradation of rRNA in the vacuole.

### 2.2 Introduction

Ribosomes are essential components of all cells and represent a major sink for cellular resources.\(^1\) The turnover of ribosomes is important to maintain cell viability and homeostasis during periods of nutritional stress. It was recognized early that macromolecular turnover and ribosomal RNA (rRNA) turnover in particular are activated when eukaryotic cells are subjected to deficiencies in carbon, nitrogen, or phosphate; available evidence pointed to a role for autophagic processes in the delivery of macromolecules to vacuoles in plants or lysosomes in animals and protozoa for degradation and recycling.\(^2\)-\(^5\)

Autophagy is a macromolecular degradation pathway that functions in the vacuolar degradation and recycling of cellular components. Macroautophagy is the best-characterized form of autophagy and is well studied in plants.\(^6\) Macroautophagy, referred to here as autophagy, involves *de novo* formation of double-membrane vesicles in the cytoplasm through the action of autophagy-related (ATG) proteins. The autophagic vesicle, called an
autophagosome, encapsulates cargo targeted for degradation and is trafficked to and fuses with the vacuole, resulting in single-membrane autophagic bodies within the lumen. The autophagic bodies are then degraded and the breakdown products recycled. Autophagy functions at a basal level to maintain homeostasis and can be upregulated during stress to aid plant survival. Both non-selective and selective autophagy pathways exist in many eukaryotes.

Recently, a selective type of autophagy termed ribophagy was described in yeast cells undergoing nitrogen starvation. Genetic analyses showed that ribophagy specifically targets ribosomes for degradation during nutritional stress in a process that depends on components of the basal, non-selective autophagy machinery, such as the scaffold protein Atg17 and E1-like protein Atg7. Mutations affecting activity of Ubp3, a ubiquitin protease, caused interruption of ribophagy but not basal autophagy and resulted in cell death when cells were maintained in prolonged nutrient starvation. This indicates that ribosome turnover through selective ribophagy is essential for survival in stressed yeast cells, a conclusion also supported indirectly by transcriptome analyses. While ribophagy has not yet been specifically studied in other eukaryotes, mounting evidence suggests that the process could be conserved in plants and animals and that starvation is not required for ribophagy to occur.

In addition to an important role in response to nutrient deficiency, rRNA turnover also seems to be necessary for maintenance of cellular homeostasis during periods of normal growth. In Arabidopsis thaliana mutants lacking RNS2 (At2g39780), a non-specific endoribonuclease of the RNase T2 family that resides in the endoplasmic reticulum and the vacuole, rRNA has a longer half-life than in wild-type (WT) plants, indicating that this enzyme is necessary for normal rRNA decay. Moreover, the rns2-2 mutants accumulated RNA intracellularly and mutant cells accumulated acidic vesicles revealed by monodansylcadaverine (MDC) staining,
hypothesized to be autophagosomes, even under non-starvation conditions. These results led to the hypothesis that RNS2 participates in a ribophagy-like mechanism that targets ribosomes for degradation as a housekeeping function that maintains cellular homeostasis. Disruption of this mechanism would lead to accumulation of RNA and the induction of non-selective autophagy as a compensatory mechanism.\textsuperscript{19} This mechanism seems to be conserved in eukaryotes. Mutations affecting \textit{Danio rerio} (zebrafish) RNASET2 also resulted in animals that accumulate rRNA in lysosomes, particularly in neurons, and this accumulation caused white matter lesions in the fish brain, again indicating that rRNA degradation is necessary for cellular homeostasis in non-starvation conditions.\textsuperscript{18}

The involvement of Arabidopsis RNS2 and zebrafish RNASET2 in housekeeping rRNA turnover is consistent with the hypothesis that the conservation of the RNase T2 family in all eukaryotes is due to a conserved housekeeping role performed by these enzymes.\textsuperscript{20} In fact, RNase T2 enzymes have been shown to participate in rRNA degradation in conditions of nutritional stress in addition to the housekeeping role in plants and animals described above. For example, RNase T2 enzymes accumulate in the vacuoles of phosphate-deprived tomato cells in parallel with significant rRNA degradation;\textsuperscript{2} Rny1, the only RNase T2 enzyme in yeast, is responsible for rRNA and bulk RNA degradation in response to starvation and oxidative stress which results in metabolic changes;\textsuperscript{5, 21, 22} and mutations affecting \textit{Tetrahymena thermophila} RNase T2 enzymes cause changes in the patterns of rRNA degradation observed under starvation, suggesting that these enzymes also participate in a ribophagy-like mechanism as a nutrient stress response.\textsuperscript{23}

While there is some agreement that rRNA degradation can occur in the vacuole or lysosome in plants and animals, the mechanisms of transport of rRNA or ribosomes to these
organelles is not well characterized, with only the ribophagy mechanism in yeast being somewhat understood. The analysis of rns2-2 phenotypes led us to hypothesize that autophagy may be part of this process. In this work we establish that the rns2-2 mutant has increased basal autophagy. Higher levels of RNA were demonstrated to accumulate in rns2-2 and atg mutants. rRNA accumulated in vacuoles from rns2-2 and rns2-2atg9 plants, but not rns2-2atg5 mutants. Both RNS2 and autophagy therefore participate in an RNA turnover mechanism, and this autophagic turnover of rRNA is dependent on ATG5 but not ATG9, suggesting a differential role for these proteins.

2.3 Results

2.3.1 ATG8-labeled autophagic bodies accumulate in an rns2-2 mutant

The Arabidopsis thaliana RNS2 null mutant (rns2-2) was previously shown to accumulate MDC-stained structures even under normal nutrient-rich conditions,\(^1\) which were hypothesized to be autophagosomes. Since this assumption is central to the hypothesis that ribosomes are recycled through a ribophagy-like mechanism in Arabidopsis, and since MDC can stain other acidic organelles in addition to autophagosomes,\(^2\) our first objective was to determine whether the MDC-stained structures observed in rns2-2 are autophagosomes. For this purpose, we used transgenic WT and rns2-2 plants containing green fluorescent protein (GFP)-ATG8e (At2g45170). ATG8 isoforms decorate both the inner and outer surface of a completed autophagosome.\(^6\),\(^8\),\(^25\) Fusion of this protein with GFP allows visualization of autophagosomes present in the cytoplasm and autophagic bodies present in the vacuole; thus GFP-ATG8 has been used extensively as an autophagosome and autophagic body marker.\(^26\)-\(^28\)
It was unknown if the potential accumulation of autophagosomes observed in 
*rns2*-2 was a result of increased autophagosome formation or decreased autophagic body 
degradation in the vacuole. To differentiate between these two possibilities, vacuolar degradation 
was inhibited with concanamycin A (ConA). ConA inhibits V-type ATPases and subsequently 
raises vacuolar pH, which reduces hydrolytic enzyme activity and blocks hydrolase delivery to 
the vacuole.\textsuperscript{29, 30} Seven-day-old seedlings were incubated in liquid Murashige and Skoog (MS) 
medium containing either 1μM ConA or dimethylsulfoxide (DMSO) as a solvent control and 
incubated for 5 hours in the dark on an orbital shaker. Seedling roots were then imaged using 
confocal microscopy (Fig. 1). WT/*GFP-ATG8e* roots had few GFP-labeled autophagosomes 
without treatment and little autophagic body accumulation upon treatment with ConA. *rns2-
2/GFP-ATG8e* also had a low number of autophagosomes visible, likely because GFP-ATG8e-
labeled autophagosomes are difficult to detect in the cytoplasm due to the presence of free 
cytosolic GFP-ATG8e. However, upon treatment of *rns2-2/GFP-ATG8e* plants with ConA, 
numerous GFP-labeled autophagic bodies accumulated inside the vacuole, indicating that 
autophagosomes are produced constitutively in this mutant. A similar phenotype was also seen in 
WT and *rns2-2* Arabidopsis leaf protoplasts transiently expressing *GFP-ATG8e* (Fig. S1). This 
demonstrates that the MDC-stained structures previously reported to accumulate in *rns2* mutants 
are ATG8-tagged autophagosomes, and that their accumulation results from increased 
autophagosome formation and not decreased autophagic body degradation.

### 2.3.2 Constitutive autophagy in *rns2*-2 is complemented by expression of RNS2

To confirm that the constitutive autophagy present in *rns2*-2 results from loss of RNS2 
activity, we introduced the RNS2 cDNA driven by a cauliflower mosaic virus 35S constitutive 
promoter into the *rns2*-2 mutant and assessed autophagy by staining with MDC. We first
confirmed that GFP-ATG8e colocalizes with MDC-stained autophagosomes in roots as it does in leaf protoplasts. It has recently been shown that GFP-ATG8e labeled autophagosomes do not colocalize with MDC-stained structures in Arabidopsis root tips. We typically avoid imaging the extreme root tip due to higher background fluorescence. In the region of the root in which we observe autophagosomes, between the elongation zone and ~1cm from the root tip, the majority of MDC- and GFP-ATG8e-labeled puncta co-localized in the cytoplasm (Fig S2).

Seven-day-old transgenic seedling roots from two independent transformants were stained with MDC to detect autophagosomes and visualized by fluorescence microscopy. Expression of RNS2 in rns2-2 plants led to a decrease in autophagy for both transgenic events tested (Fig. 2A and B). RNS2 activity was verified in the transgenic lines by an in-gel ribonuclease activity assay using high-molecular weight torula yeast RNA as substrate. Even though RNS2 event #1 had much lower RNS2 activity than WT, both lines showed complementation of the constitutive autophagy phenotype, indicating that relatively low RNS2 activity is sufficient for complementation (Fig. 2C).

2.3.3 Blocking autophagy in rns2-2 results in rosette growth defects but not stress tolerance defects

We hypothesized that autophagy increases to compensate for lost RNS2 “housekeeping” activity in the rns2-2 mutant. Thus, blocking autophagy in rns2-2 could lead to more severe phenotypes. To test this hypothesis, we crossed rns2-2 with the autophagy mutant atg9 to generate an rns2-2atg9 double mutant. ATG9 (At2g31260) is probably involved in trafficking membrane lipid to newly forming autophagosomes and is required for autophagosome formation. A previously described atg9 null mutant had early bolting and senescence, as well as reduced seed production and accelerated chlorosis during nitrogen starvation. We identified
an additional \textit{atg9} allele from the SALK T-DNA insertion mutant collection (SALK\_145980, CS859902), \textit{atg9-4}. Characterization of individuals homozygous for the \textit{atg9-4} mutation using MDC staining (Fig. S3) showed that, consistent with the previously described \textit{atg9} mutant, they are deficient in autophagy activation during salt, nitrogen starvation, and carbon starvation stresses.

\textit{rns2-2} was also crossed with an \textit{atg5-1} mutant (corresponding to the \textit{At5g17290} locus) to generate a \textit{rns2-2atg5-1} double mutant.\textsuperscript{28} Mutations in \textit{ATG5} have been shown to block macroautophagy, selective macroautophagy of protein aggregates, and microautophagy mechanisms in plants and other eukaryotes.\textsuperscript{28, 37-41}

\textit{rns2-2} has a T-DNA insertion in the fifth intron and is null for \textit{RNS2} RNase activity.\textsuperscript{19} To verify that \textit{rns2-2atg9-4} and \textit{rns2-2atg5-1} plants lack \textit{RNS2} activity, total protein extracts from seedlings were subjected to the in-gel ribonuclease activity assay (Fig S4A and S5A). In this zymogram analysis, multiple bands for \textit{RNS2} are sometimes detected in WT extracts due to glycosylation of \textit{RNS2}.\textsuperscript{19} In contrast, \textit{rns2-2}, \textit{rns2-2atg9-4}, and \textit{rns2-2atg5-1} lacked activity bands in the \textit{RNS2} range. RT-PCR demonstrated that expression of \textit{ATG9} and \textit{ATG5} is absent in \textit{atg9-4}, \textit{atg5-1}, \textit{rns2-2atg9-4}, and \textit{rns2-2atg5-1} mutants (Fig. S4B and S5B).

Many Arabidopsis autophagy mutants have smaller rosettes,\textsuperscript{42} while no detailed analysis of changes in growth has been performed for the \textit{rns2-2} mutant. To determine whether growth differences exist between the different genotypes analyzed in our work, twenty-six-day-old rosettes were analyzed by Rosette Tracker software.\textsuperscript{43} Rosette area was determined for rosettes of each genotype and normalized to WT (Fig. 3A and C). It is evident from this analysis that the \textit{rns2-2}, \textit{atg9-4}, and \textit{atg5-1} mutations have an effect on plant growth. The \textit{rns2-2} mutant showed a small but significant increase in total rosette area with respect to WT plants. \textit{atg9-4} and \textit{atg5-1}
were both smaller than WT as expected from observations of other \textit{atg} mutants. \textit{rns2-2atg9-4} and \textit{rns2-2atg5-1} had smaller rosette areas than \textit{atg9-4} and \textit{atg5-1} single mutants, indicating that loss of RNS2 enhances the \textit{atg} mutant growth phenotype. The \textit{rns2-2atg5-1} double mutant showed the most severe phenotype, potentially because of a more complete autophagy block than in \textit{atg9-4} mutants.\textsuperscript{44}

Autophagy mutants, including \textit{atg5-1}, have increased sensitivity to nutrient stress.\textsuperscript{28, 36, 45, 46} To test if \textit{rns2-2}, \textit{atg9-4}, \textit{atg5-1}, \textit{rns2-2atg9-4}, or \textit{rns2-2atg5-1} also showed increased sensitivity to nutrient deficiency, we subjected seedlings to fixed-carbon starvation stress.\textsuperscript{47} Seedlings were grown under a long-day (LD) photoperiod on solid MS medium lacking sucrose for 2 weeks. Seedlings were then placed in darkness for 10 days and allowed to recover for 12 days under LD. The number of surviving seedlings was compared to the number of germinated seedlings that existed prior to dark exposure (\textbf{Fig. 3B and D}). No significant difference existed between WT and \textit{rns2-2}. However, \textit{atg9-4} and \textit{rns2-2atg9-4} showed increased sensitivity to fixed-carbon starvation. No significant differences existed between \textit{atg9-4} and \textit{rns2-2atg9-4}, suggesting that autophagy is not essential for \textit{rns2-2} plant’s survival under fixed-carbon starvation and that \textit{rns2-2} adopts \textit{atg9}-like phenotypes in the \textit{rns2-2atg9-4} double mutant. \textit{atg5-1} containing mutants were more sensitive to fixed-carbon starvation than \textit{atg9-4} mutants (\textbf{Fig. S6}). No seedlings survived in either \textit{atg5-1} or \textit{rns2-2atg5-1}, suggesting ATG5 is more important than ATG9 for surviving fixed-carbon starvation stress. This is consistent with previous studies using the \textit{atg5-1} mutant.\textsuperscript{28} The difference in percent survival of WT and \textit{rns2-2} seedlings between \textit{atg9-4} - and \textit{atg5-1}-containing studies is likely due to differences in the seed stocks used in each analysis.
2.3.4 Autophagy is blocked in *rns2-2atg9-4* and *rns2-2atg5-1*

Some autophagy-like processes have been described that do not rely on the classical autophagy machinery.⁴⁸⁻⁵⁰ To characterize the dependence of the *rns2-2* constitutive autophagy phenotype on known core autophagy proteins, we analyzed autophagy in *rns2-2atg9-4* and *rns2-2atg5-1* double mutants. Seven-day-old seedling roots were stained with MDC to detect autophagosomes and were visualized by confocal microscopy (Fig. 4A and B). Autophagy is present at a low basal level in WT seedlings,⁵¹,⁵² as indicated by a few fluorescent puncta present in root cells. *rns2-2* has increased autophagy, with multiple puncta.¹⁹ As determined above, *atg9-4* and *atg5-1* are deficient in autophagy, and no puncta were observed. Introduction of the *atg9-4* or *atg5-1* mutant alleles eliminated the increased autophagy phenotype in *rns2-2*. Quantification of the puncta observed with MDC staining and fluorescence microscopy showed that autophagosome number is significantly and substantially increased in *rns2-2* and significantly reduced in *atg9-4*, *atg5-1*, *rns2-2atg9-4*, and *rns2-2atg5-1* (Fig. 4C and D). More important, mutations in *ATG9* and *ATG5* completely eliminate the constitutive autophagy phenotype in the *rns2-2* mutant. Differences in autophagosome number are seen in the *rns2-2* mutant in different experiments, possibly due to variability in growth conditions, compared to WT seedlings. These results further confirm that the MDC-stained puncta that accumulate in *rns2-2* are autophagosomes, and that this accumulation relies on the core ATG machinery.

As an alternative approach to test for accumulation of autophagic bodies (and other vesicles) in the vacuole, seedlings were treated with either 1μM ConA or DMSO as a solvent control for 8 hours in liquid MS medium in darkness. Differential interference contrast microscopy (DIC) was used to visualize vesicle accumulation in the vacuole in seedling root cells. WT, *rns2-2*, *atg9-4*, and *rns2-2atg9-4* lines all lacked vesicle accumulation upon treatment
with DMSO solvent control (Fig. S7) suggesting vesicle turnover is not affected. However, treatment with ConA resulted in some vesicles accumulating in WT, likely a result of basal autophagy, and a large number of vesicles accumulating in *rns2*-2. Very few vesicles accumulated in *atg9*-4 and *rns2*-2*atg9*-4, further indicating that autophagosome trafficking to the vacuole is blocked in the *atg9*-4 background.

### 2.3.5 Autophagosomes in *rns2*-2 contain RNA and ribosomes

We hypothesized that the increased autophagy in *rns2*-2 may be an attempt to compensate for the loss of RNS2 activity by trafficking ribosomes or rRNA to the vacuole for degradation. To investigate the role of autophagy in trafficking RNA to the vacuole, seven-day-old WT and *rns2*-2 seedlings were co-stained with MDC to visualize autophagosomes and SYTO RNAsSelect to label RNA (Fig. 5A). Some cell autofluorescence is detected in unstained controls in the MDC channel, but no puncta are observed in either WT or *rns2*-2. Significantly more MDC- and SYTO- stained bodies were observed in *rns2*-2 than in WT seedlings. Co-localization of MDC- and SYTO RNAsSelect-labeling was also significantly increased in *rns2*-2, with ~55% of the MDC-labeled structures co-localizing with the SYTO stain (Fig. 5B and C). This suggests that many of the MDC-stained bodies, most likely autophagosomes, contain RNA.

WT and *rns2*-2 seedling roots were also analyzed by transmission electron microscopy. Autophagosomes contain cytoplasmic components and have a characteristic double-membrane morphology when in the cytoplasm. Upon fusion with the tonoplast, single-membrane autophagic bodies are released into the vacuole lumen. DMSO-treated control seedlings had vacuoles lacking vesicles (Fig. 6). Treatment with ConA resulted in some accumulation of vesicles in the vacuoles of both WT and *rns2*-2 seedlings. Vesicles in WT were only found occasionally and contained little cytoplasmic cargo. *rns2*-2 cells accumulated numerous single
membrane vesicles within the vacuole that contained components such as mitochondria, endoplasmic reticulum, and ribosomes, indicating that they were autophagic bodies. No double membrane autophagosomes were observed in the cytoplasm, likely because of their transient nature and rapid trafficking to the vacuole.

2.3.6 RNA differentially accumulates in mutant lines

Previous results indicated higher levels of SYTO RNASelect staining in Arabidopsis rns2-2 roots and protoplasts. Mutations affecting a recently described selective autophagy process (RNautophagy) in mice resulted in increased total cellular RNA levels. To examine whether the total level of RNA present in rns2-2, atg9-4, atg5-1, rns2-2atg9-4, and rns2-2atg5-1 lines differs from WT, tissue was lyophilized and RNA extracted from each mutant, quantified and normalized to dry weight. rns2-2, atg9-4, atg5-1, rns2-2atg9-4 and rns2-2atg5-1 mutants were all found to have significantly higher levels of RNA than WT (Fig. 7). Interestingly, rns2-2atg5-1 had significantly higher total RNA than the single mutants and the rns2-2atg9-4 double mutant. This indicates that both RNS2 and the autophagy pathway are required for maintenance of normal cellular RNA levels, with ATG5 having a greater involvement than ATG9 in an rns2-2 background.

2.3.7 rRNA accumulates in vacuoles of rns2-2 and rns2-2atg9-4

As rRNA is the most abundant form of RNA in cells and the half-life of rRNA is increased in rns2-2, we hypothesized that rRNA can be transported to the vacuole by autophagy for degradation by RNS2. To test this hypothesis we developed a method for the quantification of RNA within the plant cell vacuole. Vacuoles were purified from WT, rns2-2, atg9-4, atg5-1, rns2-2atg9-4, and rns2-2atg5-1 plants and purity assessed by immunoblotting (Fig. S8). Vacuole preps showed high enrichment of the vacuolar protein aleurain, whereas the
endoplasmic reticulum protein BiP was undetectable. RNA was extracted from each vacuole sample, cDNA synthesized, and quantitative real-time PCR (qRT-PCR) was used to measure the amount of ribosomal RNA in each vacuole preparation. However, no reference gene RNA for qRT-PCR normalization is available within vacuoles. Therefore, to control for vacuole yield and normalize the qRT-PCR results, acid phosphatase activity was measured in aliquots of purified vacuoles (Fig. 8A). Acid phosphatase is a vacuolar resident enzyme in plant cells\textsuperscript{54,55} and has similar activity in WT, \textit{rns2-2, atg9-4, atg5-1, rns2-2atg9-4}, and \textit{rns2-2atg5-1} vacuoles (Fig. 8B). Arabidopsis 18S and 25S rRNA-specific primers were used to quantify the amount of rRNA in vacuoles per unit of acid phosphatase activity. \textit{rns2-2} vacuoles were found to contain significantly more 18S and 25S rRNA than WT, \textit{atg9-4, atg5-1}, or \textit{rns2-2atg5-1} (Fig. 8C). Interestingly, disrupting autophagy via the \textit{rns2-2atg9-4} mutant did not reduce the amount of rRNA in the vacuole compared with the \textit{rns2-2} single mutant, while the \textit{rns2-2atg5-1} double mutant had reduced rRNA accumulation. Furthermore, \textit{atg5-1} vacuoles had significantly lower amounts of rRNA than WT or \textit{atg9-4}. This indicates differing functions of ATG9 and ATG5 in the trafficking and turnover of rRNA in the vacuole by autophagy.

2.4 Discussion

The cell closely monitors the synthesis and assembly of ribosomes through a number of processes due to the sizeable anabolic requirements and importance of making stable ribosomes.\textsuperscript{1,56} Several pathways have been described for the decay of mRNA, misassembled ribosomal subunits, and nonfunctional ribosomes.\textsuperscript{56,57} However, decay mechanisms by which turnover of normal ribosomes occur are not fully understood. The purpose of this work is to better understand the relationship between autophagy and the turnover of ribosomes in plants.
We show that the core autophagy machinery is activated to compensate for lost RNS2 activity in the *rns2-2* mutant, and that the autophagosomes contain RNA and ribosomes. The lack of RNS2 activity in this mutant leads to the accumulation of undigested rRNA within the vacuole, and this accumulation is blocked in an *rns2-2atg5-1* double mutant. Moreover, *rns2-2, atg9-4, atg5-1, rns2-2atg9-4*, and *rns2-2atg5-1* mutants show elevated amounts of total RNA, suggesting that autophagy is a mechanism of ribosome turnover.

RNase T2 enzymes represent the most widely distributed of all the endoribonuclease families and are conserved in eukaryotes, some prokaryotes, and some viruses.\textsuperscript{20, 58-61} RNS2 is one of five RNase T2 enzymes found in Arabidopsis and is the only class II RNase T2 constitutively expressed throughout the plant and throughout development.\textsuperscript{60, 62} RNase T2 proteins are targeted to organelles of the secretory pathway, including the endoplasmic reticulum, Golgi apparatus, vacuole, lysosome, and associated vesicles, or secreted from the cell.\textsuperscript{20, 59, 63} RNS2 is localized to the endoplasmic reticulum and vacuole,\textsuperscript{19} where it functions in degradation of rRNA (and possibly other types of RNA) after uptake into the vacuole either as free RNA or as intact ribosomes. This pathway is thus complementary to cytoplasmic RNA degradation pathways, in which different classes of ribonucleases and degradation machineries function.\textsuperscript{64, 65} Studies in multiple systems have found RNS2 orthologs to be important for the degradation of rRNA. Mutant alleles of *RNASET2* are associated with accumulation of undigested rRNA within lysosomes in zebrafish and human neurons,\textsuperscript{18, 66} and ribosomal RNA is a cellular substrate for the RNase T2 enzymes of unicellular eukaryotes such as yeast and *Tetrahymena* during nutrient starvation.\textsuperscript{22, 23}

We previously showed that under normal growth conditions *rns2-2* and anti-sense RNS2 plants had rRNA with extended half-life, indicating that rRNA is a substrate for RNS2 in plants.
Labeling with the RNA-specific stain SYTO RNASelect suggested that RNA accumulated in the vacuoles of rns2-2 mutants. To determine whether this RNA inside the vacuole included rRNA and to measure this accumulation, we developed a method to quantify rRNA within plant cell vacuoles. qRT-PCR is typically normalized to a reference gene such as 18S, actin, or Tip41-like; however, these transcripts are not present in the vacuole and therefore cannot be used as normalization controls in our system. We also tested for the presence of Ubiquitin and RNS2 but could not detect these transcripts in the vacuole fractions. Instead, acid phosphatase enzyme activity was used to determine the relative amount of purified vacuoles for standardization of qRT-PCR. With this method, we found that 18S and 25S rRNAs accumulate to ~6-fold higher levels in rns2-2 than WT vacuoles. Thus, our results demonstrate that RNS2 participates in the degradation of rRNA that has been delivered to the vacuole either by mechanisms that rely on the core autophagy machinery or by alternative mechanisms (see below).

It was previously shown that rns2-2 plants have increased accumulation of MDC-stained acidic vesicles under normal growth conditions, and we show here that these structures are in fact autophagosomes, based on the presence of ATG8 in their membranes and the requirement for ATG9 and ATG5 for their formation. Furthermore, we have shown that autophagosomes and autophagic bodies accumulating in rns2-2 contain RNA and/or ribosomes. These results support the hypothesis that autophagy is being activated to deliver rRNA to the vacuole through a ribophagy-like mechanism to compensate for a reduction in rRNA turnover due to lost RNS2 activity. Quantification of RNA in rosette leaves also indicated that autophagy may function in normal bulk RNA turnover, as rns2-2, atg9-4, atg5-1, rns2-2atg9-4, and rns2-2atg5-1 all showed increased levels of total cellular RNA.
However, total RNA accumulation in \textit{rns2-2atg5-1} double mutants was significantly higher than in single mutants, with \textit{rns2-2atg9-4} also showing a marginal increase in total RNA accumulation, suggesting that RNS2 and autophagy do not function entirely in the same pathway for RNA degradation. Reduced accumulation in \textit{rns2-2} relative to \textit{rns2-2atg5-1} and \textit{rns2-2atg9-4} may be an effect of the constitutive autophagy phenotype in \textit{rns2-2}. It is currently unknown if ribosomes can be selectively targeted by autophagy in plants. However, the autophagosomes that form in \textit{rns2-2} may also encapsulate stress granules and P-body-like structures in the cytoplasm, which are known to contain RNA degradation machineries including ribonucleases.\textsuperscript{68,69} Studies in mammals have shown that stress granules are cleared by autophagy.\textsuperscript{70} If this is the case, the constitutive autophagy may traffic cytoplasmic ribonucleases to the vacuole where they can degrade some of the RNA present there, albeit at a reduced rate since the vacuolar environment is likely below their pH optimum, and the RNases themselves may be targets for degradation by vacuolar proteases.

Interestingly, genetically blocking autophagy using the \textit{atg9-4} mutant did not eliminate the accumulation of rRNA in vacuoles, as the \textit{rns2-2atg9-4} mutant contained approximately the same amount of rRNA in its vacuoles as \textit{rns2-2}. It was recently reported that the \textit{atg9} mutant has reduced but not completely blocked autophagic flux.\textsuperscript{44} Although our microscopy data suggests that \textit{atg9-4} blocks the formation of autophagosomes, vacuolar rRNA quantification indicates that rRNA is still delivered to the vacuole in this mutant. \textit{atg5-1} is thought to completely lack autophagy, and vacuolar rRNA accumulation is completely lost in \textit{rns2-2atg5-1}. These data indicate that either \textit{atg9-4} has a low level of autophagy activity remaining that is sufficient to deliver rRNA to the vacuole but is not detectable with our microscopy assays, or that ATG9 and ATG5 function differently in the autophagic trafficking of rRNA to the vacuole.
During macroautophagy, ATG9 is involved in trafficking membrane to the phagophore during autophagosome biosynthesis while ATG5 functions in the conjugation of ATG8 to phosphatidylethanolamine. However, other forms of autophagy have been described in eukaryotes. One form, microautophagy, occurs through invagination of the vacuolar membrane to form single membrane vesicles that enter the vacuole for degradation. Microautophagy has been reported during plant senescence and development, but little is known about its role in plant cell homeostasis. It is possible that ATG5 is involved in a microautophagy-like or other novel mechanism that does not require ATG9 (Fig. 9). Thus, the atg5-1 mutant would block both macro- and microautophagy while atg9-4 would block only macroautophagy. This would also explain the lower vacuolar rRNA accumulation of atg5-1 compared with WT and atg9-4.

While it is logical to propose that the upregulation of autophagy in rns2-2 is a compensation mechanism for lost RNS2 activity that helps to maintain cellular homeostasis, the actual cellular changes caused by lack of rRNA recycling that lead to increased autophagic activity are unclear. Basal autophagy is always present in plants, turning over cytoplasmic components and functioning as a quality control mechanism. Autophagy is upregulated during the developmental processes of senescence and cell death as well as during biotic and abiotic stresses such as oxidative stress, drought, nutrient starvation, and pathogen infection. Upregulation of autophagy in rns2-2 could be caused by a lack of ribosome turnover that results in the accumulation of toxic products and oxidative stress, or it could be the result of a starvation response, since rRNA represents a large proportion of cellular resources. The turnover of ribosomes through autophagy or other mechanisms may be important for homeostasis and the resources recycled through this process, such as bases, nucleosides, nitrogen, or energy, may be needed to maintain housekeeping functions in normal cells.
In summary, we have shown that autophagy is upregulated in response to a mutation in the RNS2 ribonuclease in Arabidopsis and that the autophagosomes produced contain RNA and/or ribosomes. Disrupting either RNS2 activity or autophagy results in RNA accumulation within the cell, suggesting a role for ATG5-mediated autophagy in RNA turnover. Autophagy, however, may not be the sole process for rRNA turnover in plants. Recent reports suggest RNA and DNA can be transported into lysosomes by a transmembrane transporter in mammalian cells.\textsuperscript{53,82} LAMP2C, a splice variant of LAMP2, is a lysosomal membrane protein that binds to both DNA and RNA and facilitates transport of DNA and RNA into the lysosomal lumen. Interestingly, LAMP2-deficient mice mutants have constitutive autophagy, suggesting that autophagy may compensate for loss of the LAMP2C transporter.\textsuperscript{83} Thus, LAMP2C may serve a role in homeostasis and act in a mechanism of normal RNA turnover. Although no homolog of LAMP2C is present in Arabidopsis, possible alternative transport pathways for RNA are under investigation.

2.5 Materials and Methods

2.5.1 Plant growth and Arabidopsis thaliana genotypes

\textit{Arabidopsis thaliana} Columbia-0 accession was used as wild-type control. For microscopy analyses, seeds were surface sterilized in 33\% (v/v) bleach, 1\% Triton X-100 for 20min followed by cold treatment for \(\geq 2\) days. Plants were grown for seven days under long day (LD) conditions (16hr light/8hr dark) at 22\(^\circ\)C on nutrient solid Murashige-Skoog medium with vitamins (MS)(MSP09; Caisson Labs), 1\% sucrose, 2.4mM MES pH5.7, and 0.8\% (w/v) phytoagar (PTP01; Caisson Labs) as described.\textsuperscript{84} Plants used for protoplast, vacuole, and dry weight analyses were grown in soil under LD conditions at 22\(^\circ\)C. Sensitivity to fixed carbon
starvation was performed as described.\textsuperscript{45, 47} Seedlings were grown in LD on solid MS medium as above without sucrose for 14 days, transferred to darkness for 10 days, and allowed to recover in LD for 12 days, and the percent surviving seedlings counted. Rosette size was analyzed using 26-day-old rosettes grown in LD and Rosette Tracker software as described.\textsuperscript{43} For salt stress, seven-day-old seedlings were transferred to liquid MS medium +160mM NaCl for 6 hours. For nitrogen and carbon starvation, seven-day-old seedlings were transferred to medium lacking either nitrogen or sucrose for 4 days. Carbon starvation plates were kept in the dark as described.\textsuperscript{85, 86}

T-DNA insertion mutants used in this study were \textit{rns2-2} (SALK\_069588), \textit{atg9-4} (SALK\_145980, ABRC stock #CS859902), and \textit{atg5-1} as described by Thompson et al.\textsuperscript{28} Crossing of \textit{rns2-2} with \textit{atg9-4} and \textit{atg5-1} was done according to Weigel and Glazebrook\textsuperscript{87} Genomic DNA from F1 and F2 progeny was analyzed by PCR using the primers listed in Table S1 to identify \textit{rns2-2atg9-4} and \textit{rns2-2atg5-1} homozygous double mutant lines. GFP-ATG8e transgenic plants have been previously described.\textsuperscript{88} Wild-type \textit{RNS2} was amplified from cDNA prepared from wild-type leaves. The PCR product was inserted into the MCS11 binary vector containing a 35S promoter using the Clontech InFusion HD recombination based cloning kit (638909; Clontech Laboratories, Inc.). Both \textit{rns2-2} GFP-ATG8e and \textit{rns2-2} \textit{RNS2} expressing transgenic plants were generated by \textit{Agrobacterium tumefaciens}-mediated transformation using floral dip.\textsuperscript{89}

\textbf{2.5.2 RT-PCR and ribonuclease activity analysis}

RNA was extracted from seven-day-old seedlings using Trizol reagent (15596018; Invitrogen) and treated with DNaseI (18068015; Invitrogen). cDNA was synthesized using SuperScript III reverse transcriptase (18080093; Invitrogen) and oligo dT primer (C1101;
Promega). RT-PCR was completed for 28 cycles using the newly synthesized template and primers listed in Table S1. Ribonuclease activity assays were performed as previously described using purified high-molecular weight torula yeast RNA (R-6625; Sigma) as substrate.19,32

2.5.3 Transient transformation of rosette leaf protoplasts

Arabidopsis rosette leaf protoplasts were prepared and transformed according to Sheen90 Thirty micrograms of GFP-ATG8e plasmid DNA was used for each transformation. Transformed protoplasts were treated with 1μM concanamycin A (C9705; Sigma) or dimethyl sulfoxide (DMSO) as a solvent control for 16 hours in darkness rotating on an orbital shaker at 50rpm and visualized using confocal microscopy.

2.5.4 Staining and microscopy

For differential interference contrast microscopy (DIC), seven-day-old seedlings were treated with 1μM concanamycin A or DMSO as a solvent control in liquid MS medium for 5 hours in the dark at 50rpm on an orbital shaker. Samples were visualized using a Zeiss Axioplan II light microscope equipped with an Axio Cam HRC digital imaging system and 40x objective (Carl Zeiss Inc.). For MDC staining, seven-day-old Arabidopsis seedlings were stained with 50μM monodansylcadaverine (MDC; 30432; Sigma) in phosphate buffered saline pH 7.4 according to Contento et al.26 For all seedling microscopy, roots were imaged in the late elongation zone and neighboring cells in the differentiation zone. Root tips and older differentiation region cells were excluded. MDC fluorescence was visualized using a UV lamp and 4’,6-diamidino-2-phenylindole-specific filter (DAPI) with excitation of 360±20nm and emission of 460±25nm (Chroma Technology Corp.). The number of motile MDC-stained autophagosomes in all cells visible in the focal plane was quantified and expressed per frame.
Confocal microscopy was performed using a Leica (Leica Microsystems) SP5 X MP confocal multiphoton microscope and HPX PL APO CS 63.0x1.40 oil objective. Confocal microscopy of MDC staining used an excitation/emission of 405nm/430-550nm. For confocal microscopy of seedlings expressing GFP-ATG8e, seven-day-old seedlings were treated with 1μM concanamycin A or DMSO as a solvent control in liquid MS medium for 5 hours in the dark at 50rpm on an orbital shaker, and visualized using an excitation of 484nm and emission of 500-600nm.

For co-staining using SYTO RNaselect (S32703; Invitrogen) and MDC, seven-day-old seedlings were stained with 5μM SYTO RNaselect in liquid MS medium for 3 hours in darkness on an orbital shaker followed by three 5min washes with liquid MS medium. SYTO-stained seedlings were then subjected to MDC staining as above. Fluorescence was visualized using confocal microscopy with an excitation/emission of 405nm/430-480nm and 490nm/515-540 for MDC and SYTO RNaselect respectively with sequential excitation and acquisition to reduce cross-excitation. Fluorescence analysis and quantification was done using FIJI software.\textsuperscript{91}

2.5.5 Transmission electron microscopy

Seven-day-old Arabidopsis seedlings were collected and fixed with 2% glutaraldehyde (w/v) and 1% paraformaldehyde (w/v) in 0.1M cacodylate buffer, pH 7.2 for 48 hours at 4°C. Samples were rinsed 3 times in 0.1M cacodylate buffer and then post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hour at room temperature. The samples were rinsed in deionized distilled water and enbloc stained with 2% aqueous uranyl acetate for 30 min., dehydrated in a graded ethanol series, cleared with ultra-pure acetone, infiltrated and embedded using Spurr’s epoxy resin (Electron Microscopy Sciences). Resin blocks were polymerized for 48 hours at 65°C. Thick and ultrathin sections were made using a UC6 ultramicrotome (Leica).
Thick (1µm) sections were collected onto slides, stained with 1% toluidine blue and imaged using a Zeiss Axioplan II light microscope (Carl Zeiss Inc). Ultrathin (60-70nm) sections were collected onto copper grids and counter-stained with 2% uranyl acetate in deionized distilled water for 30 min. Images were captured using a JEOL 2100 scanning and transmission electron microscope (Japan Electron Optic Laboratories) at the Iowa State University Microscopy and Nanoimaging Facility.

2.5.6 Dry weight total RNA quantification

Arabidopsis rosette leaves were lyophilized for 2-3 days, and RNA was extracted from 4-8 mg of dry tissue using TRI Reagent (AM9738; Invitrogen) and resuspended in 100μL of RNase-free water.\textsuperscript{53} RNA was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific) and normalized to dry weight.

2.5.7 Vacuolar rRNA quantification

Vacuoles were purified from Arabidopsis rosette leaves grown under long day conditions during early bolting\textsuperscript{92} and frozen in liquid nitrogen. Vacuole purity was assessed by immunoblotting using antibodies against the endoplasmic reticulum resident protein BiP (Enzo Life Sciences, AD1-SPA-818, 1:1000 dilution) and the vacuolar protein aleurain (1:500 dilution).\textsuperscript{93} Upon thawing, vacuoles were homogenized by vortexing and 100μl used for acid phosphatase analysis. RNA was extracted from the remaining vacuoles using an RNeasy Plant Mini Kit (74904; Qiagen). For this, 900μl of vacuoles were combined with 600μl of lysis buffer RLT (Qiagen) and RNA extracted according to the manufacturer’s protocol. Samples were DNase-treated on the Qiagen RNA column using an on-column DNase (79254; Qiagen) and RNA eluted with 30μl of supplied nuclease-free water.
A 13μl aliquot of vacuole RNA was used for cDNA synthesis using an iScript Select cDNA synthesis kit (170-8896; Bio-Rad, Hercules, CA, http://www.biorad.com) using random primers with a final cDNA dilution volume of 80μl. cDNA was tested by semi-quantitative PCR using the primers in Table S1. qRT-PCR was carried out using SYBR-Green (AB-1158/A; Thermo) and a Stratagene Mx4000 multiplex quantitative PCR system (Agilent Technologies) with primers listed in Table S1, with TIP41-like used as an internal control in protoplasts. The efficiency for each primer pair and the relative amount of RNA in each sample were calculated using a standard curve as described. qPCR results were normalized to the vacuole marker enzyme acid phosphatase activity, which was measured as previously described. Three to five biological replicates per genotype were carried out.

2.6 Acknowledgements

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2.7 References


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Figure 1. GFP-ATG8e-containing autophagic bodies accumulate in *rans*2-2 seedling root cells. Seven-day-old transgenic Arabidopsis WT and *ms*2-2 seedlings expressing the GFP-ATG8e autophagosome marker were treated for 5h with either dimethyl sulfoxide (DMSO) as a solvent control or 1μM ConA to block vacuolar degradation. Roots were visualized by confocal microscopy. Images are representative of three biological replicates. Scale bar = 10μm.
**Figure 2.** Genetic complementation of *rns2*-2 mutant with wild type RNS2 eliminates the increased autophagy phenotype.

(A) Seven-day-old WT, *rns2*-2, 35S::RNS2 *rns2*-2 event 1 (RNS2 E1), and 35S::RNS2 *rns2*-2 event 2 (RNS2 E2) Arabidopsis seedling roots were stained with 50μM monodansylcadaverine (MDC) and imaged using fluorescence microscopy. Scalebar = 20μm. (B) Activity gel of RNS2 activity. Total protein extracts from seven-day-old seedlings were analyzed using an RNase activity in-gel assay. The position of RNS2 is indicated. (C) Quantification of autophagy. MDC-stained structures as shown in part (A) were quantified from fluorescence microscopy images. Autophagosomes (ATGs) were counted per frame from 30 root images taken in the late elongation zone per genotype across 3 biological replicates. Error bars represent standard error. Similar letters indicate no significant difference according to pairwise Student’s two-sided equal variance *t*-test (P>0.05).
Figure 3. Effect of the $rns2$-2 and $atg9$-4 mutations on rosette size and carbon starvation tolerance.

(A) Twenty-six-day-old Arabidopsis rosettes from WT, $rns2$-2, $atg9$-4, $atg5$-1, $rns2$-2$atg9$-4, and $rns2$-2$atg5$-1 plants grown under LD. Scale bar = 2cm. (B) WT, $rns2$-2, $atg9$-4, and $rns2$-2$atg9$-4 seedlings were grown on MS medium lacking sucrose for 14 days in LD, placed in darkness for 10 days, and recovered in LD for 12 days. (C) Boxplot of normalized rosette area (A) using Rosette Tracker software as a FIJI plugin. 54-66 plants were measured for each genotype, with 4 biological replicates for $atg9$-4 mutants and two biological replicates for $atg5$-1
mutants. The shaded box represents the interquartile range, horizontal line represents the median, and open circles represent outliers >1.5 standard deviations away from the mean. (D) Percent survival of seedlings grown under sucrose starvation conditions (B) was calculated by counting green seedlings compared to the number of germinated seedlings. 60-122 seedlings were used for each genotype, with seven biological replicates. Error bars represent standard error. Similar letters indicate no significant difference according to pairwise Student’s two-sided equal variance t-test (P>0.05).
Figure 4. *rns2-2* has constitutive autophagy that is eliminated in the *atg9-4* and *atg5-1* backgrounds.

(A-B) Seven-day-old WT, *rns2-2, atg9-4, atg5-1, rns2-2atg9-4,* and *rns2-2atg5-1* Arabidopsis seedling roots were stained with 50μM MDC and imaged using confocal microscopy. Scalebar = 25μm. (C-D) MDC-stained structures as shown in part (A-B) were quantified from fluorescence microscopy images. Autophagosomes (ATGs) were counted per frame from 30 root images taken in the late elongation zone per genotype with 3-4 biological replicates. Error bars represent standard error. Similar letters indicate no significant difference according to pairwise Student’s two-sided equal variance t-test (P>0.05).
Figure 5. MDC-stained bodies in rns2-2 co-localize with SYTO RNASelect-stained RNA.

(A) Seven-day-old WT and rns2-2 Arabidopsis seedlings were co-stained with 50μM MDC and 5μM SYTO RNASelect and analyzed by confocal microscopy. Arrowheads indicate co-localized puncta. Scale bar = 25μm. (B) Co-localization analysis by line fluorescence tracing of pixel intensity using FIJI software of co-localized puncta in rns2-2. (C) Quantification of MDC, SYTO RNASelect, and co-localized puncta. Percentage of co-localized puncta was calculated by determining the number of co-localized puncta/number of MDC-stained puncta. 5-15 images per genotype were analyzed with 3 biological replicates. Error bars represent standard error. Similar letters indicate no significant difference according to pairwise Student’s two-sided equal variance t-test (P>0.05).
**Figure 6.** *rns2-2* accumulates autophagic bodies containing organelles inside the vacuole.

WT and *rns2-2* Arabidopsis seedlings were treated for 8h in the dark with dimethyl sulfoxide (DMSO) as a solvent control or 1μM concanamycinA (ConA) to block vacuolar degradation and imaged by transmission electron microscopy. Insets show autophagic bodies within the vacuole (V). *rns2-2* inset labels: (R) ribosomes, (M) mitochondria, (ER) endoplasmic reticulum. All scale bars = 2μm.
Figure 7. Total RNA content is elevated in the *rns2-2*, *atg9-4*, and *atg5-1* mutants.

Total RNA was extracted from lyophilized adult rosette leaves. After quantification, RNA content was normalized to the mass of lyophilized tissue used for the extraction. For each genotype, five samples with leaves pooled from four plants each were collected in three sets of independently grown plants. Error bars represent standard error. Similar letters indicate no significant difference according to pairwise Student’s two-sided equal variance *t*-test (*P* > 0.05).
Figure 8. *rns2-2* and *rns2-2atg9-4* accumulate ribosomal RNA within vacuoles while *atg5-1* mutants do not.

(A) Vacuolar rRNA quantification protocol. Protoplasts were prepared from adult rosette leaves. Vacuoles were purified from these protoplasts, and checked for quality under the microscope. Each vacuole sample was then split for RNA quantification (including RNA extraction, DNase treatment, cDNA synthesis and qPCR) or acid phosphatase analysis. (B) Acid phosphatase activity is similar across all tested genotypes. Protein was extracted from leaf tissue from the same stage as those used in vacuole purification and assayed for total protein using Bradford analysis or for acid phosphatase activity using 4-methylumbelliferyl phosphate. Total acid phosphatase activity was normalized to total protein revealing no significant differences between genotypes. (C) Quantification of vacuolar ribosomal RNA. Ribosomal RNA from WT and mutant vacuoles was measured using qPCR, and the results were normalized to acid phosphatase activity to account for differences in the amount of vacuoles in each sample. These results are expressed relative to the WT average. WT, *rns2-2*, *atg9-4*, and *rns2-2atg9-4* were analyzed in triplicate for five biological replicates. WT, *rns2-2*, *atg5-1*, and *rns2-2atg5-1* were analyzed in triplicate for three biological replicates. Error bars represent standard error. Similar letters indicate no significant difference according to pairwise Student’s two-sided equal variance t-test (P>0.05).
Figure 9. Model for the role of RNS2 and autophagy in rRNA turnover.

(A) Under normal conditions RNS2 is present in the vacuole and ribosomes are in the cytoplasm with basal autophagy functioning to maintain homeostasis. (B) Mutating rns2-2 results in increased activation of autophagy. This results in accumulation of rRNA within the vacuole. This may occur either by transfer of intact ribosomes as shown, or via transport of rRNA after disassembly of ribosomes (not shown). Some of the autophagosomes may non-specifically engulf stress granules and P-body-like structures that contain RNases and contribute to some RNA degradation in the vacuole (shown in grey). (C) Disruption of autophagy in atg mutants causes accumulation of RNA in the cytoplasm, likely from ribosomes. Some ribosomes may enter the vacuole through a microautophagy-like mechanism (shown in grey). (D) rns2-2atg9-4 mutants have increased accumulation of RNA, and much of the rRNA accumulates in the vacuole, possibly by a microautophagy-like mechanism in which ATG9 is not involved. This results in some autophagic flux to the vacuole (shown in grey). (E) rns2-2atg5-1 blocks both macro- and micro- autophagy mechanisms resulting in total RNA and rRNA accumulation in the cytoplasm.
Figure S1. GFP-ATG8e-labeled autophagic bodies accumulate in *rns2-2* rosette leaf protoplasts. Protoplasts were prepared from wild type and *rns2-2* rosette leaves, transformed with 30μg *GFP-ATG8e* plasmid DNA and incubated overnight. Protoplasts were then treated with either DMSO as a solvent control or 1μM concanamycinA (ConA) to block vacuolar degradation, and visualized by confocal microscopy. Scale bar = 10μm.
Figure S2. Colocalization of GFP-ATG8e and MDC-stained vesicles in rns2-2 35S::GFP-ATG8e transgenic lines. Seedlings of the indicated genotypes were grown for seven days, stained with MDC, and visualized by confocal microscopy. Imaging was restricted to the region between the root elongation zone and ~1cm beyond the elongation zone. Three separate root images are shown as examples. Scalebar =20μm and =10μm in insets. Arrowheads within insets illustrate co-localized puncta.
Figure S3. *atg9-4* is defective in autophagy under stress conditions.

Seven-day-old Arabidopsis seedlings were subjected to salt stress, nitrogen deficiency, or sucrose deficiency, stained with 50μM MDC and imaged using fluorescence microscopy. Scalebar = 10 μm.
Figure S4. The *rms2-2atg9-4* double mutant lacks RNS2 activity and *ATG9* gene expression. (A) Activity gel of RNS2 activity. Total protein extracts from seven-day-old seedlings were analyzed using an RNase activity in gel assay. The position of RNS2 is indicated. Double bands exist due to glycosylation. Two progeny lines for *rms2-2atg9-4* were analyzed. (B) RT-PCR of *ATG9* expression using 18S rRNA and minus reverse transcriptase as controls.
Figure S5. The *ms2-2atg5-1* double mutant lacks RNS2 activity and *ATG5* gene expression.  

(A) Activity gel of RNS2 activity. Total protein extracts from seven-day-old seedlings were analyzed using an RNase activity in gel assay. The position of RNS2 is indicated. (B) RT-PCR of *ATG5* expression using 18S rRNA as a positive control
**Figure S6.** Effect of the *atg5-1* and *rms2-2atg5-1* mutations on carbon starvation tolerance.

(A) WT, *rms2-2*, *atg5-1*, and *rms2-2atg5-1* seedlings were grown on MS medium lacking sucrose for 14 days in LD, placed in darkness for 10 days, and recovered in LD for 12 days. (B) Percent survival of seedlings grown under sucrose starvation conditions (A). 22-43 seedlings were used for each genotype, with six biological replicates. Dwarf seedlings that germinated late and did not show significant root growth were excluded from quantification. Error bars represent standard error. Similar letters indicate no significant difference according to pairwise Student’s two-sided equal variance t-test (P>0.05).
Figure S7. Increased intravacuolar vesicle accumulation in \textit{rns2-2} but not \textit{atg9-4} mutants upon treatment with concanamycin A.

Seven-day-old WT, \textit{rns2-2}, \textit{atg9-4}, and \textit{rns2-2atg9-4} seedlings were treated for 8h in the dark with dimethyl sulfoxide (DMSO) as a solvent control or 1μM concanamycinA (ConA) to block vacuolar degradation. Roots were visualized using differential interference contrast microscopy (DIC). Insets show intravacuolar vesicle accumulation. Scale bar = 20μm and 10μm (insets).
**Table S1.** Primers used in this study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
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<td>TCCTGGTCTTTAATTGGGCCCGG</td>
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<tr>
<td>At25S rRNA (qRT-PCR)</td>
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<tr>
<td>ATG9 (genotyping, RT-PCR)</td>
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<td>CAAGCGGATTTCTTGGGAATG</td>
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<tr>
<td>atg9-4 (genotyping)</td>
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<td>CAAGCGGATTTCTTGGGAATG</td>
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<tr>
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<td>ATG5 (RT-PCR)</td>
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<tr>
<td>RNS2 (cloning)</td>
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<tr>
<td>rns2-2 (genotyping)</td>
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CHAPTER 3

CELL GROWTH AND HOMEOSTASIS ARE DISRUPTED IN ARABIDOPSIS RNS2-2 MUTANTS MISSING THE MAIN VACUOLAR RIBONUCLEASE ACTIVITY

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3.1 Abstract

3.1.1 Background and Aims

Enzymes belonging to the RNase T2 family are essential for normal ribosomal RNA turnover in eukaryotes. In *Arabidopsis thaliana* this function is performed by RNS2. The null mutant *rns2-2* has increased rRNA half-life and constitutive autophagy. Here we aim to determine the molecular changes that take place in the *rns2-2* mutant that may lead to altered cellular homeostasis, manifested by the observed cellular phenotype.

3.1.2 Methods

To determine the effect of defective rRNA turnover on cellular homeostasis, we used comparative transcriptome and metabolome analyses of 10 day old wild-type and *rns2-2* seedlings to identify molecular processes affected in the mutant. Bioinformatics analyses suggested additional phenotypes that were confirmed through direct plant size measurements and microscopy.

3.1.3 Key Results

Few genes were differentially expressed in the *rns2-2* mutant, indicating that control of autophagy in this genotype is mainly achieved at the posttranscriptional level. Among differentially expressed genes we identified transcripts related to carbon flux processes, particularly the pentose phosphate pathway, and metabolite analyses confirmed changes in the levels of PPP intermediates. We also found differentially expressed genes related to cell wall loosening and found a decrease in monosaccharide components of cell wall hemicellulose. As a potential effect of weaker cell walls, *rns2-2* plants are larger than wild-type controls, due to larger cells and increased water content.
3.1.4 Conclusions

We propose that lack of rRNA recycling in rns2-2 cells triggers a change in carbon flux, which is redirected through the pentose phosphate pathway to produce ribose-5-P for de novo nucleoside synthesis. rRNA or ribosome turnover is thus essential to maintain nucleoside homeostasis.

3.2 Introduction

As ribosomes account for a substantial proportion of cellular resources, ribosome turnover is a critical factor in maintaining homeostasis, particularly during nutrient deficiency [1-4]. When yeast cells are subjected to starvation conditions, ribosomes are targeted for degradation through autophagy-related processes, in which cargo is taken up into the vacuole for degradation and recycling. A selective autophagy pathway termed ribophagy targets ribosomes in nitrogen-starved yeast cells [5]; ribosomes are preferentially targeted to the autophagic pathway compared with other cytoplasmic proteins under these conditions. The ubiquitin deconjugation enzyme Ubp3p and its cofactors Bre1p, Cdc48, and Ufd3 [6] are essential factors for selective autophagy of 60S (but not 40S) ribosomal subunits [5]. A possible role in ribophagy for the ubiquitin ligase Rsp5 has also been proposed [7], whereas the Ltn1 E3 ligase antagonizes ribophagy activation [8]. Non-selective autophagy has also been implicated in the degradation of ribosomes in yeast cells upon starvation [9].

The mechanism of autophagic RNA degradation was recently dissected in yeast [9]. After transport of ribosomes to the vacuole through either ribophagy or non-selective autophagy, rRNA is hydrolyzed by Rny1, a member of the RNase T2 family [10], which is the main vacuolar ribonuclease (RNase) activity in yeast [9, 11]. The turnover of rRNA yields 3’-NMPs
that are then converted to nucleosides by the vacuolar non-specific phosphatase Pho8. These nucleosides are transported back to the cytoplasm where they are further broken down by the nucleosidases Pnp1 and Urh1 to produce purine and pyrimidine bases that are either reused by the cell or secreted [9].

The turnover of functional ribosomes under non-stress conditions is less well-characterized. In Arabidopsis thaliana, the vacuolar RNase RNS2, also a member of the RNase T2 family, is necessary for normal rRNA decay. Arabidopsis mutants lacking RNS2 activity have higher levels of total RNA [12], and rRNA has a longer half-life in rns2 mutants than in wild-type (WT) plants [13]. rRNA accumulates in the vacuole in the rns2-2 mutant and this accumulation depends on the presence of the core autophagy protein ATG5 but not the core autophagy protein ATG9 [12]. Interestingly, lack of RNS2 activity results in an increased basal autophagy phenotype, with mutant cells displaying a level of autophagy under normal conditions similar to that observed in WT plants only under stress conditions [12, 13]. These results suggested that normal ribosomes or rRNA are targeted for vacuolar degradation by a selective autophagic process, likely similar to ribophagy [14]. Once in the vacuole, rRNA is hydrolyzed by RNS2. We hypothesized that lack of rRNA degradation in the rns2 mutants leads to an imbalance in cellular homeostasis that triggers constitutive autophagy as a compensatory mechanism [12, 13].

This normal rRNA decay mechanism seems to be conserved in other eukaryotes. Zebrafish have two RNASET2 genes but only one is constitutively expressed [15]. This protein resides in lysosomes, and mutant zebrafish lacking RNASET2 activity also show accumulation of rRNA in the lytic organelle [16]. Similar accumulation was observed in human cells in which RNASET2 expression was knocked down with artificial miRNAs [16]. In both cases, electron
microscopy showed that lysosomes are full of dense material, likely rRNA. This accumulation causes changes in cellular homeostasis that is particularly prominent in neuronal cells, and leads to white matter lesions in the brain, associated with familial cystic leukencephalopathy [16, 17].

The constitutive autophagy phenotype observed in the Arabidopsis rns2-2 mutant could be the result of different changes in cellular homeostasis. Since ribosomes are an important sink of cellular resources, lack of rRNA degradation could lead to a change in the energy balance or the availability of nitrogen in the cell. Alternatively, the phenotype could result from a decrease in available purine and pyrimidine bases. To gain insight into the type of imbalance that triggers constitutive autophagy in the rns2-2 mutant, we analyzed changes in the transcriptome caused by the rns2-2 mutation and complemented this analysis with metabolome studies. We found a small number of differentially expressed genes in rns2-2, which indicated that the pentose phosphate pathway (PPP) and cell wall processes are affected in the mutant. We also observed changes in the level of some of the sugars that participate in the PPP and in the sugar composition of the cell wall. Finally, we determined that the rns2-2 mutant is larger than WT plants due to an increase in cell growth, which could be caused by an increase in carbon availability and could explain the changes in cell wall observed in our analysis. Our results suggest that the mutant may use the PPP to divert C flux toward production of ribose-5-P, an essential substrate for the de novo synthesis of nucleosides, and that constitutive autophagy in the mutant is likely triggered as a compensatory mechanism in response to a deficiency in the nucleoside pool.
3.3 Materials and Methods

3.3.1 Plant material for microarray and metabolite analysis, and RNA preparation

Seeds of *Arabidopsis thaliana* ecotype Columbia-0 and the *rns2*-2 mutant were sterilized and stratified overnight as previously described (Hillwig et al 2011). Seeds were plated on agar plates with Murashige and Skoog (MS) modified basal medium with Gamborg vitamins (Phytotech, M404). Plants were grown under 16hr light-8hr dark with a light intensity of ~120 μmol m$^{-2}$s$^{-1}$ at 60% humidity and 21° C. Ten day old seedlings were then collected and frozen in liquid N$_2$. Seedlings were ground with a mortar and pestle under liquid N$_2$ and RNA was extracted from 50-100 μg of ground tissue using the Qiagen RNeasy plant mini kit (Qiagen 74903). RNA was then treated with TURBO DNase (Life Technologies, AM2238) to remove contaminants genomic DNA. RNA samples were tested for quality using the Agilent 2100 Bioanalyzer and only samples with an RNA integrity number (RIN) higher than 7 were used for microarray analysis.

3.3.2 Microarray analysis

RNA (250 ng) from *rns2*-2 mutant and WT seedlings was labeled using the GeneChip®3’ IVT Expression kit (Affymetrix, 901229) and hybridized to the Affymetrix Arabidopsis ATH1 Genome Array GeneChip (Affymetrix, 900385) using an Affymetrix hybridization kit (Affymetrix, 900720). Arrays were scanned on a GeneChip® scanner 30007G. Raw intensity data were generated by the Affymetrix Expression console software. Three independent biological replicates were analyzed for each genotype. RNA labeling, hybridization, and scanning were performed by the Microarray facility at Iowa State University.

Data were normalized using Robust Multi-array Average (RMA) [18]. Data were further analyzed to find differentially expressed genes using the affyLM GUI in R and a $p$-value cutoff
of 0.005 [19]. The list of differentially expressed genes was analyzed for over-represented Gene Ontology (GO) terms using Virtual Plant’s BioMaps analysis with a p-value cutoff of 0.01 [20]. Network analysis was completed using Virtual Plant’s Gene Networks analysis and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, both the primary and secondary subtypes, allowing for zero hops [21]. The returned network analysis was further modified in Adobe Photoshop for visual clarity. Raw and normalized data were deposited in the GEO database (http://www.ncbi.nlm.nih.gov/geo/) to allow for public access (accession number GSE81218).

3.3.3 qPCR

cDNA was synthesized from DNase-treated RNA using the qScript Flex cDNA kit (Quanta, 95049). qPCR was performed using the Perfecta SYBR Green Supermix (Quanta, 95070) on a Stratagene MX4000, using 30 ng of sample cDNA template, and relative quantification was completed using a standard curve [22]. As a loading control to normalize the qPCR data, we used a transcript previously identified to be stably expressed, the TIP41-like (At4g34270) transcript (forward primer sequence 5’CCGGCGATTCAGATGGAGACGG3’ and reverse 5’TGCTGAGACGGCTTGCTCCTG3’) [23]. Microarray targets verified by qPCR included AT3G45970, Arabidopsis thaliana expansin-like A1 (forward primer 5’ GAGTTTCTTCCGGGACA 3’, reverse primer 5’ ATCGCAAGGAACCTGCTTGT 3’); AT2G41640, glycosyl transferase (forward primer 5’ TGTGCTTCAAACGTCACCCA 3’, reverse primer 5’ TGCGAAACGAATCTAGGAGGG 3’); AT1G79530, Glyceraldehyde 3-Phosphate Dehydrogenase of Plastid 1 (forward primer 5’ ATGGGGTTACAGCAACCGAG 3’, reverse primer 5’ CACGGGCAAGCTAAGGTG 3’); and AT1G16300, Glyceraldehyde 3-
Phosphate Dehydrogenase of Plastid 2 (forward primer 5' ATGGGGTTACAGCAACCGAG 3', reverse primer 5' ACGTTGCGGGATATGTTT 3').

3.3.4 Metabolite analysis

Ten-day-old seedlings were grown on Murashige-Skoog Vitamin and Salt mixture phytoagar plates with 16 h light/8 h dark at 22°C. Non-targeted metabolite profiling was carried out using gas chromatography-mass spectrometry (GC-MS). Twenty mg of sample tissue were extracted using 350 µL hot methanol. Samples were incubated at 60°C for 10 min followed by sonication for 10 min. 350 µL chloroform and 300 µL water were added, samples mixed by vortexing, and polar and non-polar fractions separated by centrifugation. Both fractions were retained separately for metabolite analysis and dried in a nitrogen evaporator followed by a speed-vac concentrator. Samples were methoximated using 50 µL of 20 mg mL⁻¹ methoxamine hydrochloride in dry pyridine at 30°C for 1.5 h with shaking. Samples were treated with 70 µL bis-trimethyl silyl trifluoroacetamide with 1% trimethylchlorosilane for 30 min at 65°C, dried under nitrogen, dissolved in 100 µL pyridine, and analyzed on an Agilent 7890A-GC using an HP5ms column equipped with a 5975C MSD detector (Agilent Technologies). Temperature was raised from 70°C to 320°C at a rate of 5°C min⁻¹ with a 2 mL min⁻¹ helium flow rate. The gradient terminated at a hold at 320°C. Ionization voltage was 70 eV and interface temperature was 280°C. Data were deconvoluted and analyzed using the Automatic Mass Spectral Deconvolution and Identification System (AMDIS) [24] and metabolites were identified using mass spectra referenced to authentic standards in the Iowa State University W.M. Keck Metabolomics Research Laboratory and the National Institute of Standards and Technology 05 mass spectra library [25].
Pentose Phosphate Pathway metabolites were quantified from plants grown as for non-targeted analysis. Approximately 30 mg of fresh tissue from whole seedlings was collected and ground in liquid nitrogen. Samples were extracted with methanol/chloroform/water (2.5:1:1 v/v/v) followed by methanol/chloroform (1:1 v/v) [26]. Samples were combined and water was added, followed by centrifugation. Samples were then dried to a tenth volume. Extracts were labeled with aniline (Sigma) and EDC (N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide) (Sigma) as previously described [27, 28]. Standards (Sigma) were labeled with aniline-\(^{13}\)C\(_6\) (Cambridge Isotope Laboratories) and EDC. Aniline-labeled standards and samples were analyzed by liquid chromatography-mass spectrometry (LC-MS) in an Agilent QTOF 6540 using a Zorbax Eclipse XDB-C18 column (Agilent). Elution began in water and was raised to 50% acetonitrile in 15 minutes, then raised to 90% acetonitrile in 0.1 min with a flow rate of 0.8 mL min\(^{-1}\) while acquiring chromatograms in negative ion mode between \(m/z\) 100-1200. Data acquisition and processing was completed using the Agilent MassHunter software. Both GC-MS and LC-MS analyses were carried out at the Iowa State University W.M. Keck Metabolomics Research Laboratory.

### 3.3.5 Growth and cellular phenotype analysis

For rosette measurements, 66 Arabidopsis plants of each genotype were grown on soil for 4 weeks. Basal rosettes were measured using Rosette Tracker software [29] for both diameter and area of the rosette. Water content was calculated by comparing the mass of adult basal rosette fresh tissue and after lyophilization for 4 days. Root lengths were measured on seedlings grown on vertical agar plates with MS modified basal medium with Gamborg vitamins. Roots were measured after removal from agarose and straightening on a solid surface. For WT and \(rms2\) 2 38 and 39 roots were measured representing three independent plates. The length of root
cells was measured by confocal microscopy. Five-day-old roots grown on vertical agar plates as above were gently removed from the agar and stained for 10 minutes using 10 µg mL\(^{-1}\) Oregon Green 488 Carboxylic Acid Diacetate (carboxy-DFFDA, stock 5mg mL\(^{-1}\) in acetone) (Life Technologies, O6151) and 5µg mL\(^{-1}\) propidium iodide (Molecular Probes, P3566) in half-strength MS. After three 5-min washes in half-strength MS medium, root cells just above the zone of elongation were imaged on a confocal microscope (Leica SP5 X MP). For WT and \(\text{rms}2-2\) 593 and 530 cell lengths were measured using ImageJ [30], from 36 and 25 roots respectively, taken from 5 independent plates.

### 3.3.6 Cell wall analysis

For each genotype, six or seven samples were analyzed. Four week old basal rosette tissue was ground in liquid nitrogen. Alcohol-insoluble cell wall was extracted using ethanol and acetone as previously described [31] and treated with \(\alpha\)-amylase. One mg of alcohol-insoluble cell wall was hydrolyzed using 2 N trifluoroacetic acid, and monosaccharide components were analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using a CarboPac PA-20 (Dionex) column and gradient conditions as previously described [32, 33]. Relative proportions and response factors were determined relative to a standard mixture of equimolar L-Fucose, L-Rhamnose, L-Arabanose, D-Galactose, D-Glucose, D-Xylose, D-Mannose, D-Galacturonic Acid and D-Glucuronic Acid (Sigma).
3.4 Results

3.4.1 The rns2-2 mutation causes only minor transcriptome changes in Arabidopsis seedlings

We hypothesized that comparison of gene expression in the rns2-2 mutant with gene expression changes in WT plants under different conditions could indicate how loss of ribonuclease activity resulted in disruption of homeostasis, manifest as constitutive autophagy. Thus, as a first approach to understand the molecular processes that trigger this phenotype, we performed a transcriptome analysis of WT and rns2-2 mutant seedlings grown on plates under normal conditions, using the Affymetrix Arabidopsis ATH1 Genome Array GeneChip that provides almost full genome coverage.

Initial data analysis using strict cutoffs for false discovery rate (FDR = 0.01) identified only RNS2 as a differentially expressed (DE) gene. A more relaxed analysis identified 38 transcripts differentially expressed (p<0.005) in rns2-2 seedlings (Table 1). 15 genes had increased expression (between 1.5 and 3.1 fold) in the mutant and 22 genes, in addition to RNS2, had reduced expression (between 1.9 and 5.5 fold) in the rns2-2 plants compared with WT. The small number of differentially expressed (DE) genes and the small magnitude of expression changes was unexpected, considering the strong cellular phenotype observed in the mutant [12, 13]. We selected several representative genes to confirm the microarray results by qPCR analysis (Fig 1). The expression of the expansin-like gene ATEXLAI (AT3G45970) was determined to be 36% lower in the mutant by qPCR and 52% lower by microarray, and similar results were obtained for the glycosyltransferase gene AT2G41640. The microarray probe 262939_s_at matches two homologous Arabidopsis genes, AT1G79530 and AT1G16300, corresponding to the plastidial glyceraldehyde-3-phosphate dehydrogenases GAPCP-1 and GAPCP-2. qPCR analysis
showed that \textit{GAPCP-1} expression is almost 2-fold higher in \textit{rns2-2}, close to the 2.29 fold increase observed for the probe 262939_s_at in the microarray. On the other hand, no change was observed for \textit{GAPCP-2} by qPCR.

We used Genevestigator [34] to identify stress conditions or treatments that co-regulate subsets of the \textit{rns2-2} DE genes. This analysis revealed that many DE genes in \textit{rns2-2} are also differentially regulated in plants grown under conditions that alter carbon availability (Figure 2). Most DE genes were also regulated, in the same direction, in Arabidopsis plants after transition from a sugar-restricted condition to a sugar-replete state by addition of glucose to the growth medium [35]. A similar correlation between DE in \textit{rns2-2} and response to C availability was observed in the dataset corresponding to genes DE in response to moderate C depletion followed by addition of sucrose to replenish C availability [36], with 18 genes regulated in the same direction by this treatment and in the \textit{rns2-2} mutant (data not shown, all genes are included in the set regulated by glucose addition in Fig 2). A number of \textit{rns2-2} DE genes were also regulated by carbon starvation [37, 38], particularly \textit{rns2-2}-repressed genes, although in this case the regulation observed in \textit{rns2-2} is the reverse of that observed under starvation. A large proportion of DE genes were also regulated by nicotinamide treatment, with a pattern of regulation opposite to the pattern observed in \textit{rns2-2}. Finally, a subset of \textit{rns2-2} DE genes were identified as KIN10 targets in an experiment that analyzed changes in the transcriptome of protoplasts transiently expressing a \textit{KIN10} gene controlled by a constitutive promoter [39]. KIN10 is a protein kinase that activates gene expression and metabolic pathways in response to low cellular energy levels [40].

We also carried out a Gene Ontology (GO) enrichment analysis to identify functional categories overrepresented in our dataset of DE genes. We identified a significantly enriched
(p>10^8) number of genes involved in cell wall modification and in the pentose phosphate pathway, among other terms (full list of significant over- or underrepresented GO terms is provided in [Supplementary Table 1]). Network analysis also revealed a clear node connected to the pentose phosphate pathway (PPP) and C flux (Figure 3). This set of DE genes included three induced genes (the plastidial glyceraldehyde-3-phosphate dehydrogenases GAPCP-1 and GAPCP-2 and the transketolase AT2G45290) and two repressed genes (the glucosamine/galactosamine-6-phosphate isomerase family protein gene AT1G13700 and the ketose-bisphosphate aldolase class-II family protein gene AT1G18270). AT1G18270 is interesting in that the predicted protein product has both a ketose-bisphosphate aldolase domain and a 6-phosphogluconate dehydrogenase domain, potentially linking glycolysis/gluconeogenesis with the PPP. Less-organized groups of genes related to cell-wall processes and lipid metabolism, signaling and transport were also identified. Cell wall-associated genes include several expansins (AT2G39700, AT3G29030, AT2G03090) and expansin-like (AT3G45970) genes, a glycosyl hydrolase (AT1G64390) and a glycosyltransferase (AT2G41640), and two osmotin-like genes (AT2G28790 and AT4G38660). Lipid-related genes induced include a phospholipase/carboxylesterase (AT3G15650), two phosphatidylinositol 4-kinases not differentiated by the microarray probe (AT5G64070 and AT5G09350), and a GDSL-motif lipase (AT1G74460), while repressed genes are a dihydrosphingosine phosphate lyase (AT1G27980) and two lipases (AT3G62860 and AT1G02660).

3.4.2 Metabolite analysis confirms changes in the pentose phosphate pathway and cell wall composition of the rns2-2 mutant

To confirm the metabolic changes suggested by our transcriptome analysis, we performed non-targeted metabolome profiling. Seedlings grown exactly as the material used for
transcriptome analysis were used to extract polar and non-polar metabolites. The resulting extracts were analyzed by GM-MS. Few non-polar metabolites showed differential accumulation in the \textit{rns2}-2 mutant, and none was unequivocally identified based on MS (not shown). Among the polar metabolites identified, glucose, fructose, and sedoheptulose showed reduced levels in the \textit{rns2}-2 mutant compared to WT (Fig. 4A). It is important to note that phosphate groups are lost with our analysis method; thus it is not possible to differentiate between phosphorylated and non-phosphorylated forms of these metabolites. However, these results also pointed to an effect of the \textit{rns2}-2 mutation on the PPP. We therefore performed a direct analysis of PPP metabolites using LC-MS [27, 28]. This analysis confirmed that sedoheptulose-7-P (S7P) level is significantly lower in the \textit{rns2}-2 mutant. The LC method used did not separate efficiently ribose-5-P (Ri5P), ribulose-5-P (Ru5P), and xylulose-5-P (Xu5P) but the combined peaks were also significantly lower in the \textit{rns2}-2 mutant than in WT plants (Fig. 4B).

Our transcriptome data also indicated differential regulation of cell wall modifying enzymes. Thus, we analyzed the monosaccharide composition of cell walls from WT and mutant plants. Our results showed that mannose and glucuronic acid levels are significantly lower in the cell wall of \textit{rns2}-2 plants (Fig. 5), suggesting that mutants have a potential defect in hemicellulose content [41].

3.4.3 The \textit{rns2}-2 mutation causes a growth phenotype associated with cell length

We previously observed that the \textit{rns2}-2 mutant is larger than WT plants [12]. Differential expression of genes related to cell wall modification and changes in cell wall monosaccharide composition suggested that increased cell expansion may account for this phenotype. To test this hypothesis we first confirmed that \textit{rns2}-2 basal rosettes are larger than WT rosettes (Fig 6A). We also observed that root length is increased in mutant plants (Fig 6B). The length of fully
elongated root cells was analyzed by confocal microscopy, and the root cells of the \textit{rns2-2} mutant were determined to have a small but significant increase in length relative to WT cells (avg. 100 \( \mu \text{m} \) long) (Fig 7), with \textit{rns2-2} cells being in average 5.7\( \mu \text{m} \) longer. Finally, the proportion of dry weight versus fresh weight of mutant and WT plants was determined, and \textit{rns2-2} was found to have a higher water content than WT (Fig 8).

\section*{3.5 Discussion}

Ribosome turnover is essential to maintain cellular homeostasis in Arabidopsis. Mutations that inactivate the main vacuolar ribonuclease, RNS2, led to an increase in rRNA half-life [13]. \textit{rns2} mutants accumulate higher levels of total RNA than WT plants, and 28S and 18S rRNA accumulate in mutant vacuoles, indicating that rRNA recycling mainly occurs in the plant vacuole [12]. In addition to this increase in RNA accumulation, \textit{rns2} mutants have a constitutive autophagy phenotype, indicating that RNS2 function is needed to maintain normal cellular homeostasis [12, 13]. Surprisingly, we found that this strong cellular phenotype was not accompanied by a large reprogramming of the transcriptome. A small number of genes showed differential expression in our microarray analysis, and none was part of the core autophagy machinery, even though we have shown that at least \textit{ATG5} and \textit{ATG9} are necessary for the autophagy phenotype displayed by \textit{rns2} plants [12]. Remarkably, about 20\% of the genes DE in the \textit{rns2-2} mutant have been identified as targets of KIN10 regulation, indicating that the KIN10 pathway is repressed in the mutant. KIN10 is a central integrator of energy signaling in plants [42], activating multiple signaling pathways as a response to low energy levels.

Consistent with the KIN10 comparison, our transcriptome analysis suggests that the \textit{rns2-2} plants are not in a nutritional deficit status, as DE genes show regulation consistent with high levels of energy, based on the comparison with starvation or glucose/sucrose replenishing
treatments. However, it is clear that carbon flux is affected in the mutants, as both expression of genes in the PPP pathway and PPP metabolites showed altered levels. The inverse correlation between regulation of DE genes in the \textit{rns2-2} mutant and regulation by nicotinamide treatments also deserves attention. Nicotinamide is a byproduct of NAD degradation and in plants it is normally salvaged to synthesize new NAD [43, 44]. In other organisms, nicotinamide treatments cause an increase in NAD\textsuperscript{+} and NADP\textsuperscript{+} levels and decrease the redox ratio (NADH/total NAD) [45-47], and the same effect could be expected in plants. Thus, inverse regulation by the \textit{rns2-2} mutation and nicotinamide treatments would suggest that the mutant has an elevated level of NAD(P)H, also consistent with a high energy state.

Most of the reactions catalyzed by enzymes encoded by DE genes associated with carbon flux identified in our analysis are reversible, and changes in steady-state levels of metabolites are difficult to interpret because a decrease in an intermediary metabolite could equally be explained as a reduction in the activity of the pathway, or as an increase in its activity with increased consumption of intermediates. However, the indication that \textit{rns2-2} mutants are not “starved” allows us to hypothesize a putative flux for the PPP [\textbf{Supplementary Fig 1}]. Our model suggests that the oxidative phase of the PPP is downregulated (also suggesting that NADPH levels are high), while the non-oxidative phase is activated to channel glyceraldehyde-3-P and fructose-6-P towards the production of ribose-5-P. The same conditions would favor production of glyceraldehyde-3-P by glyceraldehyde-3-P dehydrogenase, further pushing carbon flux to the generation of ribose-5-P. By analogy with the mechanism of rRNA recycling described in yeast cells undergoing nitrogen starvation [9], we can expect that plant rRNA is normally hydrolyzed by RNS2 in the vacuole to produce 3’MNPs that are then further processed to nucleosides by a yet uncharacterized vacuolar activity and transported to the cytoplasm for reutilization. Lack of
rRNA recycling in the *rns2-2* mutant should directly impact the nucleoside and/or nucleobase cellular pool, triggering compensatory mechanisms, including constitutive autophagy and redirection of the carbon flux for *de novo* nucleoside synthesis. Equilibrative Nucleoside Transporter 1 (ENT1) is the main exporter of nucleosides from the vacuole to the cytoplasm in Arabidopsis [48]. Transgenic plants with altered levels of this transporter show significant changes in nucleotide metabolism [48, 49], consistent with our model indicating that vacuolar RNA recycling is important to maintain nucleotide and cellular homeostasis.

We observed increased expression of several expansins in the *rns2-2* mutant. Expansins cause loosening of the cell wall, likely by disrupting non-covalent interactions between cellulose fibrils or between cellulose and xyloglucans (hemicellulose) [50]. We also found changes in the monosaccharide composition of the cell wall in mutant plants, specifically a decrease in mannose and glucuronic acid, two common components of hemicellulose [41]. Decreased levels of these sugars could reduce the degree of hemicellulose crosslinking and cause further loosening of the cell wall. Cell wall loosening results in a reduction in cell wall stress and turgor pressure, which is followed by increased water flow into the cell. This water influx elastically expands the wall until turgor and wall stress are restored, and as a result cells elongate [51]. Thus, reduced strength of the cell wall could explain the increased cell and plant size phenotypes observed in the *rns2-2* mutant, and the increase in water content in mutant plants. The reduction in cell wall monosaccharide components could also be a consequence of the alteration in carbon flux [Supplementary Figure 1]. We observed reduced levels of fructose and glucose in the mutant, and these sugars are precursors of mannose and glucuronic acid respectively [52, 53]. Our microarray results identified the glycosyl hydrolase ATGH9C2 gene (*AT1G64390*) as the top
induced gene in the mutant; *AT1G64390* was previously proposed to facilitate cell elongation [54], supporting our hypothesis.

Why do *rns2-2* mutants show a constitutive autophagy phenotype? It is clear that autophagy is connected to nutrient recycling and maintenance of cellular energy status [55-57]. It has also been shown that autophagy contributes to starch breakdown in leaves [58]. The relatively low number of genes with alterations in transcript level in the *rns2-2* mutant indicates that the regulation of the autophagy phenotype is mainly achieved at the posttranscriptional level in the mutant. There is strong evidence that autophagy is, at least in part, regulated posttranscriptionally in plants, particularly through the TOR (target of rapamycin) pathway [55, 59]. TOR is a protein kinase that phosphorylates components of the ATG1 kinase complex [60], which in turn represses autophagy activation. TOR is therefore a negative regulator of autophagy, and decreased TOR expression leads to constitutive autophagy in Arabidopsis [61]. In addition, the mammalian homolog of KIN10, AMPK, is a positive regulator of autophagy via post-transcriptional mechanisms, in pathways both upstream of and independent from TOR signaling [62]. Our microarray and metabolome results suggest, however, that the *rns2-2* mutant is in a high energy state, which should maintain TOR signaling and repress the KIN10 pathway, as also observed in our results. Thus, it is evident that the autophagy phenotype in *rns2-2* is uncoupled from the signaling pathways sensing sugar or energy status.

It is possible that a mechanism sensing a decrease in the level of nucleosides or related metabolites may trigger autophagy to increase the turnover of macromolecules needed to provide carbon backbones which can be channeled through the PPP for *de novo* nucleoside synthesis. Alternatively, autophagy could be triggered as a side effect of changes in C flux. Our analysis suggests that *rns2-2* plants might have high NADPH levels. High NADPH could result in
production of reactive oxygen species (ROS) and consequently trigger autophagy. Transformation of tobacco cells with a mutant calmodulin that hyper-activates NAD kinase results in an increase in NADPH levels and a concomitant increase in ROS accumulation through the activity of NADPH oxidase [63], indicating that NADPH oxidase is not the rate limiting step in ROS production, and that an increase in NADPH level in the rns2-2 mutant could in fact trigger production of ROS. ROS accumulation, in turn, is able to induce autophagy in plants [55].

Clearly, more detailed metabolome studies combined with genetic approaches and, eventually, direct carbon flux analyses will be necessary to fully understand the molecular bases for the rns2-2 cellular and morphological phenotypes. However, our results strongly support an essential role for rRNA recycling in maintenance of cellular homeostasis and primary metabolism, likely by providing nucleosides or related metabolites for cellular function.
3.6 Funding

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3.7 Acknowledgements

We thank Dr. Zhihong Song and Dr. Ann Perera from the W. M. Keck Metabolomics Research Laboratory at Iowa State University for GC-MS and LC-MS technical assistance and compounds identification. Andrew Severin from the Iowa State University Genome Informatics Facility provided valuable help with microarray analysis. We also thank Dr. Olga Zabotina for assistance with cell wall analysis, helpful discussions, and for critical reading of the manuscript.
3.8 References


3.9 Figures and Tables

**Figure 1. Verification of microarray results.** Expression of genes identified as DE in the microarray analysis was analyzed by quantitative RT-PCR (qPCR). RNA was extracted from WT and *rnsl2* seedlings grown identically to the material used for transcriptome analysis. Genes selected for testing were *ATEXLA1* (*AT3G45970*), *glycosyl transferase* (*AT2G41640*), *GAPCP-1* (*AT1G79530*) and *GAPCP-2* (*AT1G16300*). Results were normalized using the expression of *TIP41-like* (*AT4G34270*) as loading control and then to the average of the WT expression. The analysis was performed using four different RNA samples for each genotype. *t*-test *p* values are indicated above *rnsl2*-WT comparisons with significant difference in expression level.
Figure 2. Genes differentially expressed in the *rns2-2* mutant are regulated by the energy status of the cell. The heatmap shows the regulation of the DE genes identified in the microarray analysis and regulation by carbon starvation, glucose re-feeding, or nicotinamide treatments, each of which change the energy status of the cell, obtained from public databases and the literature. Expression in protoplasts overexpressing KIN10 is also included in the comparisons.
Figure 3. Differentially expressed genes in the rns2-2 mutant suggest changes in carbon flux and cell wall modifications. Network analysis of differentially expressed genes was carried out using Virtual Plant and the Aracyc database. Diamonds represent genes while circles represent metabolites. Not all DE genes were associated with an enriched category.
**Figure 4. Metabolite analysis indicates changes in levels of sugars in the rns2-2 mutant.**

**A.** Non-targeted metabolite analysis. Polar metabolites were extracted from WT and rns2-2 mutant seedlings grown using the same conditions applied for microarray analysis. Metabolites were separated using gas-chromatography and identified by mass-spectrometry. Only metabolites with significantly ($t$-test) different levels are shown. At least four different samples were analyzed for each genotype. **B.** Targeted metabolite analysis to identify changes in the PPP. Ten day old WT and rns2-2 seedlings were grown as in A. Metabolites were extracted, labeled using $^{13}$C, and analyzed by RPLC-MS. Significant differences ($t$-test) are indicated. Results are the average of four independent experiments with at least 3 replicates per genotype. S7P, sedoheptulose-7-phosphate; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; 6PGA, 6-phosphogluconate; Ru5P, ribulose-5-phosphate; Xu5P, xylulose-5-phosphate, Ri5P, ribose-5-phosphate.
Figure 5. Cell wall composition is affected in the *rns2-2* mutant. Seedlings were grown as described in figure 4, and cell wall was extracted. Monosaccharide composition of WT and *rns2-2* cell walls after α-amylase treatment was analyzed by HPAEC-PAD. Results were obtained as mol % and then normalized to the average of the WT level for each metabolite. Significant differences (*t*-test) are indicated. At least six independent samples for each genotype were analyzed.
Figure 6. *rns2-2* is larger than WT. A. Basal rosettes of mature (four-week-old) plants were measured for using Rosette Tracker software. Area and diameter were significantly different (*t*-test) between WT and *rns2-2* plants. 66 plants from each genotype were analyzed. B. Root length was measured in ten-day-old WT and *rns2-2* seedlings grown on vertical agar plates. *t*-test *p* value is indicated. Three independent plates containing the two genotypes were analyzed. Both plant size and root length were normalized to the average of the WT values.
Figure 7. *rns2-2* has longer root cells. A. Representative image of a root stained with propidium iodide (red) and Oregon Green (green) dyes used to measure root cell length by confocal microscopy. A WT root is shown. B. After image capture, cell lengths were measured using ImageJ. *t*-test *p* value is indicated. More than 500 cells were measure for each genotype.
Figure 8. *rns2-2* plants contain more water than WT plants. Water content was determined as the weight difference of basal rosette tissue from WT and *rns2-2* seedlings grown as in Figure 4, before and after four days of lyophilization. The ratio of dry weight/wet weight was normalized to the WT average. *t*-test *p* value is indicated. Five independent samples for each genotype were analyzed.
Table 1. List of genes differentially expressed in the rns2-2 mutant seedlings with respect to wild-type

<table>
<thead>
<tr>
<th>Locus</th>
<th>Fold change</th>
<th>P-value</th>
<th>Gene name and/or annotation</th>
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<tr>
<td>AT1G64390</td>
<td>3.11681</td>
<td>2.48E-05</td>
<td>ATGH9C2, glycosyl hydrolase</td>
</tr>
<tr>
<td>AT2G39700</td>
<td>3.09792</td>
<td>0.00044</td>
<td>ATEXPA4, expansin</td>
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<tr>
<td>AT3G29030</td>
<td>2.648013</td>
<td>0.000256</td>
<td>ATEXPA5, expansin</td>
</tr>
<tr>
<td>AT2G28790</td>
<td>2.591433</td>
<td>0.000145</td>
<td>osmotin-like protein</td>
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<td>AT3G15650</td>
<td>2.438696</td>
<td>0.000443</td>
<td>Phospholipase</td>
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<td>AT2G03090</td>
<td>2.381119</td>
<td>0.000275</td>
<td>ATEXPA15, expansin</td>
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<tr>
<td>AT1G79530;AT1G16300</td>
<td>2.289448</td>
<td>4.94E-05</td>
<td>GAPCP-1 and GAPCP-2, plastid glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>AT1G61580</td>
<td>2.278667</td>
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<td>ARP2, ribosomal protein</td>
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<tr>
<td>AT3G14210</td>
<td>2.205033</td>
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<td>0.000374</td>
<td>GDSL-motif lipase</td>
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<td>AT5G64070;AT5G09350</td>
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<td>0.00025</td>
<td>phosphatidylinositol 4-kinase</td>
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<td>0.00039</td>
<td>unknown protein</td>
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<td>AT1G01860</td>
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<td>0.000331</td>
<td>DPL1, Dihydroposphingosine phosphate lyase</td>
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<td>AT5G24800</td>
<td>0.524407</td>
<td>0.000204</td>
<td>ATBZIP9, transcription factor</td>
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<tr>
<td>AT1G18270</td>
<td>0.522557</td>
<td>0.000196</td>
<td>ketose-bisphosphate aldolase/6-phosphogluconate dehydrogenase</td>
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<td>AT3G45970</td>
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<td>0.000103</td>
<td>ATEXLA1, expansin-like glucosamine/galactosamine-6-phosphate isomerase</td>
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<td>0.000121</td>
<td>SYP122, syntaxin</td>
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<td>0.000349</td>
<td>unknown protein</td>
</tr>
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<td>AT2G41640</td>
<td>0.302148</td>
<td>0.000234</td>
<td>Glycosyltransferase</td>
</tr>
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<td>AT5G49450;AT5G49448</td>
<td>0.285898</td>
<td>0.000191</td>
<td>ATBZIP1, transcription activator</td>
</tr>
</tbody>
</table>
### Table 1 Con’t

| AT3G04640   | 0.26628 | 0.000295 | glycine-rich protein |
| AT1G05575   | 0.246055 | 0.000199 | unknown protein      |
| AT1G02660   | 0.234144 | 0.00012  | lipase class 3       |
| AT1G19530   | 0.221147 | 0.000481 | unnamed protein      |
| AT3G10930   | 0.204299 | 0.000385 | unknown protein      |
| AT5G59820   | 0.182259 | 0.000351 | RHL41, transcription factor |
| AT2G39780   | 0.025392 | 7.49E-09 | RNS2                 |
Supplemental Figure S1. Integrated model of effects of mutation of *ms2-2*

**Supplementary Table 1.** GO term analysis of transcriptome data
4.1 Abstract

Turnover of ribosomal RNA, the largest RNA component of the cell, is critical for maintenance of cell homeostasis. Normal rRNA in plants is degraded through a ribophagy-like mechanism which requires the autophagy machinery, including ATG9, and the RNase T2 family of ribonucleases, represented by RNS2 in Arabidopsis. While we have established strong evidence of this ribophagy-like mechanism in Arabidopsis, the effects of mutation of these components has not been established in adult plants. We performed a transcriptome analysis on adult basal rosette tissue of WT, rns2-2, atg9-4 and rns2-2atg9-4 plants. We found a strong differential regulation of genes involved in plant cell wall and sugar processing as well as in...
genes responsive to oxidative stress. We further examined the effects on the pentose phosphate pathway metabolites in \textit{atg9-4} and \textit{rns2-2atg9-4} and observed similar changes to those previously reported for \textit{rns2-2}. Additionally, we showed that \textit{rns2-2} plants have higher levels of reactive oxygen species and that constitutive autophagy in \textit{rns2-2} plants is dependent on the production of reactive oxygen species by NADPH oxidase. These results provide new insight into the plant response to disruption in the ribophagy-like mechanism as well as the relationship between \textit{rns2-2}, autophagy and reactive oxygen species.

4.2 Introduction

Ribosomal RNA forms up to 80\% of the RNA in eukaryotic cells, necessitating mechanisms for rRNA turnover to maintain homeostasis [1]. The RNase T2 family of ribonucleases is highly conserved in eukaryotes [2]. RNase T2 enzymes participate in rRNA decay in a number of species, including humans [3], zebrafish [3], yeast [4], Tetrahymena [5] and Arabidopsis [6, 7].

In Arabidopsis, the RNase T2 responsible for rRNA degradation is RNS2 [7]. When RNS2 is eliminated in the null mutant \textit{rns2-2}, rRNA has a longer half-life as measured through pulse-chase labeling experiments [7]. Further, staining using the RNA-specific fluorescent dye SytoRNA Select reveals accumulation of RNA in the vacuole, occasionally in puncta [7]. Loss of RNS2 results in constitutive autophagy shown by increased numbers of bodies stained by the autophagy-indicating dye monodansylcadaverine (MDC), marking autophagosomes, in \textit{rns2-2} roots [7]. Colocalization of MDC and SytoRNA Select staining reveals RNA accumulates in the autophagosome [6]. These results indicate that rRNA degradation is required to maintain cellular homeostasis.
This degradation of rRNA in plants is hypothesized to represent a ribophagy-like mechanism, similar to ribophagy in yeast. In starved yeast, ribophagy is a mechanism for the selective degradation of ribosomes which is dependent on the autophagy machinery and results in vacuolar degradation of rRNA by the yeast RNase T2, Rny1p [8]. In Arabidopsis, rRNA is transported to the vacuole by autophagosomes in a process dependent on two Arabidopsis autophagy components, ATG9 and ATG5 [6]. Both ATG9 and ATG5 are involved in rRNA degradation, as shown through bulk RNA accumulation in the null mutants for each [6]. ATG9 is necessary for autophagosome formation and atg9-4 plants have increased levels of both total and rRNA [6]. However in an rns2-2atg9-4 double mutant we see rRNA accumulation in the vacuole, indicating there is a secondary non-atg9 dependent mechanism for rRNA transport to the vacuole [6]. We observed non-vacuolar rRNA accumulation in the atg5-1 plant, leading to the conclusion that rRNA transport to the vacuole is dependent on ATG5 [6].

Previously, to investigate the constitutive autophagy and homeostasis disruption phenotype in rns2-2 we performed a transcriptome analysis on seedlings [9]. The number of differentially expressed genes were few, indicating that the homeostatic disruption observed in rns2-2 is largely controlled at the posttranscriptional level or that the microarray method used in that study was not sensitive enough to detect small variations in gene expression. However, our microarray analysis revealed a number of transcripts associated with carbon flux pathways, and a non-targeted metabolome analysis determined that there were significantly lower levels of sugars including glucose, fructose and sedoheptulose [9]. We looked more specifically at the pentose phosphate pathway components and found that sedoheptulose-7-phosphate and unresolved ribose-5-phosphate, ribulose-5-phosphate and xylulose-5-phosphate are lower in rns2-2 plants [9]. Further, expression of genes encoding for cell wall remodeling proteins and expansins was
upregulated in rns2-2 plants [9]. Cell wall monosaccharides associated with hemicellulose branching were lower in rns2-2, and together with the higher level of expansins suggest that the mutant has weaker cell walls than wild-type plants [9]. Weaker cell walls explain the phenotype of rns2-2, as rns2-2 plants are larger [6]. With weaker cell walls, there would be more cell elongation and water influx, resulting in the observed larger basal rosette and longer roots. This hypothesis was further supported by our findings showing higher water content in rns2-2 tissue and longer cell length in mutant root cells [9].

While rns2-2 plants are slightly but significantly larger than WT, the rns2-2atg9-4 mutant reveals a more pronounced plant phenotype than rns2-2, being significantly smaller than the WT and more sensitive to carbon starvation [6]. To investigate this exacerbation of homeostasis disruption in the rns2-2atg9-4, provide deeper transcriptomic coverage than in the previous microarray experiment and further examine the ribophagy-like mechanism in Arabidopsis we performed an RNA-seq analysis of the transcriptome of wild-type and mutant plants.

Furthermore, to examine the relationship between nutrient availability and maintenance of homeostasis through rRNA degradation, and to explain the greater sensitivity to nutrient starvation in the mutants, we used RNA-seq to examine the transcriptomes of adult basal rosette leaf from WT, rns2-2, atg9-4 and rns2-2atg9-4 in two growth conditions, nutritionally reduced and replete. We found overrepresentation of transcripts for cell wall and sugar modifying compounds as well as a strong representation of oxidative stress-responsive transcripts in all genotype comparisons, particularly higher levels in the list of unique DE genes in the atg9-4, rns2-2atg9-4 and rns2-2 mutants. Further, because our previous study revealed differential levels of pentose phosphate components in the rns2-2 mutant, we examined the pentose phosphate
pathway in \textit{atg9-4} and \textit{rns2-2atg9-4} and found that \textit{atg9-4} has lower levels of ribose-5-phosphate, ribulose-5-phosphate and sedoheptulose-7-phosphate while the \textit{rns2-2atg9-4} had lower levels of these as well as glucose-6-phosphate and fructose-6-phosphate. Upon DCFDA staining we observed that \textit{rns2-2} plants had significantly higher levels of reactive oxygen species while ROS levels in the \textit{atg9-4} and \textit{rns2-2atg9-4} mutants were approximately the same as in wild-type. DPI treatment to inhibit the activity of NADPH oxidase caused the loss of the constitutive autophagy phenotype in \textit{rns2-2}. These results demonstrated that the constitutive autophagy in the rRNA degradation-deficient \textit{rns2-2} mutant is ROS-dependent.

4.3 Materials and Methods

4.3.1 Plant material for RNA-seq, and RNA preparation

Seeds of \textit{Arabidopsis thaliana} ecotype Columbia-0 (WT), \textit{rns2-2}, \textit{atg9-4} and \textit{rns2-2atg9-4} were sterilized as previously described (Hillwig et al 2011). Seeds were planted on LC1 Sunshine Mix soil and grown 4 weeks under 16hr light-8hr dark with a light intensity of \(~120\mu\text{mol m}^{-2}\text{s}^{-1}\) at 60\% humidity and 21\° C. Plants were grown four per pot in 4 inch pots with 11 pots per two-foot flats. Plants were either fertilized (nutritionally replete) or unfertilized (nutritionally deprived). The fertilized treatment refers to plants grown on soil mix and fertilized weekly with 1L of 1X fertilizer (5mM KNO$_3$, 2.5mM KH$_2$PO$_4$, 2mM MgSO$_4$, 2mM Ca(NO$_3$)$_2$, 500μM FeEDTA, 70 μM H$_3$BO$_3$, 14 μM McCl$_2$, 500pM CuSO$_4$, 1 μM ZnSO$_4$, 200 pM Na$_2$MoO$_4$, 10 μM NaCl and 10pM CoCl$_2$). The unfertilized plants were grown on soil without addition of fertilizer. Nitrogen would be naturally present in the soil but become depleted with time. For each replicate basal rosette leaf tissue was collected from five separate plants and frozen in liquid N\(_2\). Two replicates of each genotype for each condition (fertilized or unfertilized)
were collected for a total of four samples per genotype representing four independent collections of five plants. Tissue was ground with a mortar and pestle under liquid N$_2$ and RNA was extracted from 50-100 mg of ground tissue using the Qiagen RNeasy plant mini kit (Qiagen 74903). RNA was then treated with TURBO DNase (Life Technologies, AM2238) to remove contaminating genomic DNA followed by testing for quality using the Agilent 2100 Bioanalyzer and only samples with an RNA integrity number (RIN) higher than 7.8 were used for RNA-seq analysis.

4.3.2 RNA-seq library preparation, sequencing and determination of differentially expressed genes

Libraries were prepared by the Iowa State University DNA Facility following Illumina's TruSeq RNA Sample Prep v2 protocol. Fragments were approximately 180bp. Samples were indexed across 2 lanes. Sequencing was conducted by the Iowa State University DNA Facility using the Illumina HiSeq 2500 on 50 bp paired end reads. Scythe, fastx_trimmer and sickle were used to remove adapter sequences and low quality or unreliable nucleotides from returned reads. Reads were mapped to the Arabidopsis genome V10 from Phytozome using tophat2 and limited to uniquely mapped reads and sorted using samtools. Returned BAM files were analyzed in R using EdgeR with a false discovery rate cutoff of q<0.05. Differentially expressed genes were generated from comparisons between each genotype to WT (rns2-2G, atg9-4G, and rns2-2atg9-4G), between nutrient replete and deplete samples (Nutrient), between WT and each genotype in the fertilized condition (rns2-2F, atg9-4F, and rns2-2atg9-4F), and between WT and each genotype in the unfertilized condition (rns2-2U, atg9-4U, and rns2-2atg9-4U).
4.3.3 Statistical Analysis

The list of differentially expressed genes was analyzed for over-represented Gene Ontology (GO) terms using Virtual Plant’s BioMaps analysis with a $p$-value cutoff of 0.01 [10]. Venn diagrams were generated using an online Venn diagram tool, Venny2.1.0 [11]. Comparison between the previous microarray and the RNA-seq studies were completed using manual inspection and Venny2.1.0. Statistical analysis of the overlap between gene lists was completed using a hypergeometric analysis at nemates.org.

4.3.4 Metabolomics Analysis

Plants used for metabolite analysis were ten day old seedlings grown on MS-agar plates as in the previous study, and pentose phosphate pathway metabolites were analyzed as previously described [9]. Student’s $t$-test was performed for statistical analysis.

4.3.5 NADPH Oxidase Inhibition and Reactive Oxygen Species Analysis

Reactive oxygen species were visualized using 2’,7’-Dichlorofluorescin diacetate (DCFDA). Seven day old seedlings were stained and washed as previously described and visualized using fluorescent microscopy [12]. Root tips were imaged at the widest portion of the root tip to maintain consistency and images were quantified using ImageJ and normalized to the WT intensity.

For inhibition of NADPH oxidase, similarly aged and grown seedlings were treated with either DMSO or 20 μM diphenyleneiodonium (DPI) [13]. Samples were then stained with MDC and autophagosomes (MDC-positive puncta) were visualized by microscopy as previously described [6]. The number of autophagosomes per frame was counted.
4.4 Results

4.4.1 General Results

In order to identify molecular processes that control the interaction between RNS2 function and the control of cellular homeostasis, we performed a transcriptome analysis on tissue from adult basal rosette leaves from WT, \(rns2-2\), \(atg9-4\), and \(rns2-2atg9-4\) homozygotes. Additionally, we compared the effects between plants grown under nitrogen restriction versus those grown with normal nitrogen to examine the effects of nitrogen depletion on mutants that have altered rRNA recycling. After processing, libraries for each sample had between 12,769,389 and 19,984,409 reads (Supplemental Table ST1). Normalization factors for each library ranged from 0.93 to 1.12. Analysis revealed a dispersion of 0.14775 and a biological coefficient of variance of 0.3844, indicating good quality for analysis. Differential gene expression was analyzed for comparison between all four samples of WT and each of the individual genotypes \((rns2-2G, atg9-4G, \text{and } rns2-2atg9-4G)\), as well as between WT and each of the individual genotypes under nutrient restriction \((rns2-2U, atg9-4U \text{ and } rns2-2atg9-4U)\) or nutrient abundance \((rns2-2F, atg9-4F, \text{ and } rns2-2atg9-4F)\). These comparisons allowed us to both examine the effect of genotype and examine the effect of genotype in light of nutrient availability. The differentially expressed genes for each comparison are presented in Supplemental File S1. Table 1 summarizes the number of differentially expressed genes which are up or down regulated for each genotype under each comparison \((G, F \text{ and } U)\) in relationship to WT. Further, an analysis was performed between all nutrient deplete compared to all nutrient replete samples \((\text{Nutrient})\), allowing for analysis of the effect of nutrient depletion on Arabidopsis (Supplemental File S1).

Presence or absence of nutrients appeared to have significant effect on the transcriptome for each genotype. Whereas under the fertilized condition, the \(atg9-4\) mutant had the most DE
genes, in the unfertilized condition rns2-2 had the most DE genes. While rns2-2atg9-4G had relatively few differentially expressed genes (7), rns2-2atg9-4F had only one differentially expressed gene, RNS2. However, clustering analysis of the heatmap revealed (Figure 1) that the atg9-4 mutant more closely resembles the rns2-2atg9-4 mutant in the direction and amplitude of change for the genes in the list of unique genes across all comparisons. These results may indicate that the atg9-4 mutation has a stronger impact on the trend of gene expression in rns2-2atg9-4 than the rns2-2 mutation. However, the effect of nutrient restriction had a greater impact on differential gene expression than the effect of any mutation or combination of mutations.

### 4.4.2 Effect of Nutrient Restriction

Nutrient restriction resulted in 3207 differentially expressed genes, 2184 upregulated and 1023 downregulated. Gene ontology analysis was performed to identify enriched biological functions. Based on these results, metabolic processes, including cell wall and specialized metabolites, photosynthesis, DNA replication, mRNA processing and cell cycle processes are disrupted under nitrogen deficiency. The electron transport chain in particular is disrupted with 11 of the 19 genes associated with the photosynthesis electron transport chain in Arabidopsis being down regulated between -1.8 and -3.08 fold under nutrient depletion. The genes include Ferric reduction oxidase 7 (AT5G49740), Photosystem II CP43 chlorophyll apoprotein (ATCG00280), Photosystem Q(B) protein (ATCG00020), Photosystem I iron-sulfur center (ATCG01060) and NAD(P)H-quinone oxidoreductase subunit H, chloroplastic (ATCG01110). These results are similar to results in nitrogen-deficient sorghum in which photosynthesis is severely reduced [14].

Lack of fertilizer addition normally results in nitrogen deficiencies in Brassicaceae. Because expected genes, such as nitrate reductase, were missing, we analyzed our microarray
data using a comparison with differentially expressed gene lists obtained from published transcriptomic analyses of different nitrogen treatments in Arabidopsis. We selected three studies with available transcriptomic analysis to allow normalization of statistical methods [15-17]. Of the studies selected, Wang et al performed a microarray analysis on the ATH1 genome array with ten day old root and shoot samples from plants grown hydroponically using ammonium as the nitrogen source followed by treatment or no treatment with nitrate [15]. Vidal et al performed their experiment using RNA-seq technology on root samples similarly treated [16]. Scheible et al studied transcriptomic effects in whole seedlings analyzed through microarray based technology on the ATH1 chip through growth of seedlings in liquid medium replete with nitrogen (C) followed by either continuing to grow in replete nitrogen (N+) or transfer to nitrogen deplete medium (N-) [17]. During our analysis, we found that there is minimal overlap between differentially expressed genes among previously published experiments. This is likely due to a number of factors including differences in platform, environment, experimental design, nitrogen source, plant tissue, and plant stage. However, our data set had a statistically significant overlap with the transcriptome data previously published by Wang et al and the global trend of differentially expressed genes appears congruous (Figure 2). These results suggest that, at least partially, nitrogen deficiency has an important contribution to the nutrient effect observed in our experiment.

4.4.3 General Effects of Mutation

Across all genotype comparisons, analysis revealed a total number of 348 differentially expressed genes (q<0.05), 80 of which are represented in two or more comparisons (Supplemental Table ST2). Comparison of atg9-4 relative to WT revealed 53 DE genes in the atg9-4G comparison, 316 genes in atg9-4F and 27 genes in atg9-4U. Analysis of rns2-2 revealed
8 DE genes in the *rns2-2G* comparison, 8 DE genes *rns2-2F* and 239 DE genes in *rns2-2U*. The relatively small number of genes in nutrient replete *rns2-2* is consistent with our previous microarray result, supporting the hypothesis that while there are significant disruptions to homeostasis in *rns2-2*, as shown through growth and carbon flux disruption and constitutive autophagy, these changes must be post-transcriptionally or post-translationally regulated. DE gene analysis of *rns2-2atg9-4* against wild type in the *rns2-2atg9-4G* comparison revealed 7 DE genes, analysis of *rns2-2atg9-4F* revealed 1 DE gene and analysis of *rns2-2atg9-4U* revealed 58 DE genes. Analysis of expression level of all transcripts indicated to be differentially expressed in one or more comparisons reveals similar expression patterns (up or down regulation) in the mutants studied (Figure 1). In order to find similarities in regulation in different mutants in the ribophagy-like mechanism, we compiled a list of differentially expressed genes for each genotype (in comparison to WT), combining differentially expressed genes from genotype only (G), nutrient replete (F) and nutrient deplete (U). Comparison between this list for each genotype reveal 14 genes shared across all genotypes, nine genes shared between *rns2-2* and *atg9-4*, 31 genes shared between *rns2-2* and *rns2-2atg9-4*, and eight genes shared between *atg9-4* and *rns2-2atg9-4* (Figure 3).

**4.4.4 Comparison to Microarray**

An analysis was performed to examine the relationship between effects of the *rns2-2* mutation in seedling plants and adult leaves. In order to achieve this, we compared the list of differentially expressed genes from our previous microarray analysis of *rns2-2* seedlings and DE genes in mature plants from the current RNA-seq experiment. Comparison of differentially expressed genes between our RNA-seq experiments including the *rns2-2* and *rns2-2atg9-4* and our previously microarray *rns2-2* results revealed a significant ($p<0.000005$) but small number
of shared genes (5). The genes shared as differentially expressed between the transcriptomic analysis of seedlings and mature plants are RNS2 (AT2G39780), the gene mutated in the rns2-2 line, ATGH9C2 glycosyl hydrolase (AT1G64390), EXPANSIN (AT3G29030), ATEXLA1 expansin (AT3G45970) and an unknown protein (AT3G10930). Analysis of the GO terms for these genes reveal overlap, particularly of the cell wall modifying and extracellular proteins, as well as the hydrolase molecular function, supporting phenotypic difference found in the mature plant.

4.4.5 Comparison Across Genotype

Due to the abundance of transcriptomic information and the core question of how plants are impacted by mutation in the ribophagy-like mechanism, the list of differentially expressed genes for each genotype comparison was analyzed for overlap. This analysis revealed overlap of a large number of oxidative stress and pathogen responsive genes. Cell growth and cell wall modifying functions may explain the pathogen responsive genes, as well as oxidative stress response. Below is individual examination of shared differentially expressed genes in these two categories.

4.4.5.1 Reactive Oxygen Species and Senescence Associated Genes

Alpha-dioxygenase 1 (AT3G01420), up regulated in atg9-4F, atg9-4U, atg9-4G, rns2-2U and rns2-2atg9-4U, is an oxidative stress response gene. An Acireductone Dioxygenase (AT2G26400) is differentially expressed in atg9-4F and atg9-4G. AT1G74590, a glutathione transferase linked to reactive oxygen species, is upregulated in atg9-4F and atg9-4G. The expression of these genes indicate the presence of oxidative stress and/or production of reactive oxygen species in the mutants, and could point to a link between ROS and the constitutive autophagy phenotype observed for rns2-2.
A large number of DE genes are also senescence associated, perhaps linking to the autophagy mutant early senescence phenotypes. Among these are two anthocyanin accumulation/biosynthesis genes, also linked to reactive oxygen species response, AACT1 (AT5G61160) and PAP2NUDT24 (AT5G19470). PAP2NUDT24 is involved in hydrolysis of nucleoside diphosphates as well as anthocyanin accumulation. These genes are upregulated in the rns2-2U but downregulated in atg9-4F. ATSWEET15, also known as Senescence Associate Gene 29, (AT5G13170) is upregulated in atg9-4F, atg9-4U, atg9-4G and rns2-2atg9-4U. This senescence associated gene encodes for a sucrose efflux protein found in the vacuole.

Senescence-associated gene 12 (AT5G45890) is a cysteine protease which is responsive to cytokinin, auxin and sugars and is upregulated in atg9-4F, atg9-4G, rns2-2U and rns2-2atg9-4U. It is induced in response to the loss of glycolic oxidase activity as well and controls pectic enzymes through processing the precursor of the active enzymes [18, 19]. Beta glucosidase 30 (AT3G60140) is a senescence associated gene differentially regulated in rns2-2atg9-4G, atg9-4F and atg9-4G.

4.4.5.2 Pathogen Associated Genes/Cell Wall Genes

Comparison across the lists of differentially expressed genes for each genotype reveals a small group of pathogen associated or cell wall modifying differentially expressed genes. These genes include a trypsin inhibitor, Arabidopsis thaliana kunitz trypsin inhibitor 1 (AT1G73260), involved in plant-pathogen response, which is upregulated in atg9-4F, atg9-4U, atg9-4G, rns2-2U, rns2-2atg9-4U and rns2-2atg9-4G. This gene is also regulated in response to hydrogen peroxide, further supporting the link to reactive oxygen species as a possible signal for the constitutive autophagy observed in rns2-2. Plant methylesterase 17 (AT2G45220) is differentially expressed in atg9-4U, atg9-4F, atg9-4G, rns2-2atg9-4U, rns2-2atg9-4G as well as
*rns2*-2U comparisons. The protein encoded by this gene, a pectin/methylesterase inhibitor superfamily gene, is found in the membrane near the plant cell wall where it modifies the cell wall. This gene is annotated to be involved in pectin catabolic processes and differential expression of this gene has been previously associated with a sugar metabolism-mediated root growth phenotype in a phosphatidylinositol monophosphate 5-kinase mutant [20]. UGT85A1 (*AT1G22400*) is another glucosyl transferase and is differentially expressed in *atg9*-4F and *atg9*-4G. The *rns2*-2F and *rns2*-2G comparisons show differential expression of a plant defensin (*AT5G44420*). Plant defensins 1.2B (*AT2G26020*), and 1.2C (*AT1G75830*) are differentially expressed in *rns2*-2F, *rns2*-2U and *rns2*-2G. These are cell wall/extracellular proteins and are induced by fungi and insects, a response which often includes cell wall modification. Similarly, *rns2*-2U and *rns2*-2G comparisons reveal differential expression of *AT4G16260*, a glycosyl hydrolase, related to the cell wall modification. ILITHYIA (*AT1G64790*), differentially expressed in *rns2*-2U and *atg9*-4G, is involved in stomatal closures by the innate immune system in response to bacteria. Additionally, the *rns2*-2U and *rns2*-2*atg9*-4U share 25 genes that have various functions but which are all down regulated, including functions such as pectin lyase, endoxylloglucan transferase, arabinogalactan protein, alcohol dehydrogenase, and DNA repair. This further highlights the enrichment of pathogen response in the mutants in rRNA degradation, as well as potentially providing further light into a previously reported phenotype of *rns2*-2, increased water content. These pathogen response genes impact cell wall modifications, further supporting the previously reported enlarged *rns2*-2 plant and cell size phenotype effect.

### 4.4.5.3 Additional Genes of Interest

Comparisons *atg9*-4F and *atg9*-4G, as well as *rns2*-2*atg9*-4G reveal differential expression for cationic amino acid transporter AAT1/caat1 (*AT4G21120*) which might be either
a response to carbon flux disruption or balancing ions, likely since ATPTR3, a peptide transporter, \((AT5G46050)\) that responds to high NaCl is also differentially expressed in \(atg9-4F\) and \(atg9-4G\). Additionally, as a transporter of nitrogenous compounds such as amino acids, this provides another potential link to disruption of nutrient homeostasis in response to mutation in rRNA degradation pathways [21]. Comparisons \(atg9-4F\), \(atg9-4G\) and \(rns2-2atg9-4G\) have differential expression of Auto-Inhibited Ca2+ ATPase 12 \((AT3G63380)\), an ATPase family protein.

### 4.4.6 Gene Ontology Analysis of Differentially Expressed Genes

Gene ontology analysis was conducted to determine the impact on biological processes of these mutations that alter the plant’s ribophagy-like mechanism (Supplemental File S3). Gene Ontology analysis of the \(rns2-2\) differentially expressed genes revealed many cell wall and extracellularly localized processes, particularly those high in xyloglucosyl transferase and hydrolase activities. Further, DE genes were enriched for gene ontologies involved in response to carbohydrate and chemical stimulus, cell wall loosening and carbon fixation. The \(atg9-4\) mutant revealed enrichment of endomembrane and extracellular associated proteins, with activity most represented by hydrolase and oxidoreductase activity. These genes were mostly associated with response to biotic stimulus and defense, compared to the GO terms of differentially expressed genes in \(rns2-2\) which were carbohydrate stimulus and chemical stimulus. Gene ontology analysis in \(rns2-2atg9-4\) revealed a high similarity to the \(rns2-2\) with an emphasis on cell wall modifying or extracellular proteins with activities for xyloglucosyl transferases and hydrolases.

While the \(rns2-2atg9-4\) mutant has too few DE genes with respect to the other genotypes for accurate comparison of GO analysis, the \(rns2-2\) and \(atg9-4\) lines can be compared. Defense response is overrepresented in all of the \(rns2-2\) and \(atg9-4G\) and \(atg9-4F\) comparisons.

4.4.7 Metabolite Analysis

The effect of the \textit{rns2-2} mutation on the pentose phosphate pathway was previously investigated in chapter 3. Here, in order to further examine the association of the ribophagy-like mechanism in plants with PPP, we expanded the analysis to the \textit{atg9-4} and \textit{rns2-2atg9-4} genotypes. When compared to WT, the \textit{rns2-2atg9-4} plant reveals a lower level of Sedoheptulose-7-phosphate (S7P), Fructose-6-phosphate (F6P), glucose-6-phosphate (G6P) and ribose-5-phosphate, xylose-5-phosphate and ribulose-5-phosphate (R5P&Xu5P&Ri5P) which were not separated by this method (Figure 4). The \textit{atg9-4} line had marginally lower levels of F6P and significantly lower levels of S7P, G6P and R5P&Xu5P&Ri5P than WT. The greater influence on the pentose phosphate pathway by the \textit{rns2-2} and the \textit{atg9-4} mutation in \textit{rns2-2atg9-4} is consistent with the smaller size and greater sensitivity to carbon deprivation observed for this mutant.
4.4.8 Reactive Oxygen Species and Autophagy

The high numbers of differentially expressed genes which are associated to oxidative stress or reactive oxygen species signaling led us to examine reactive oxygen species production in WT, *rns2*-2, *atg9*-4, and *rns2*-2*atg9*-4 using DCFDA staining. We observed a significant (P<0.05) increase in the levels of reactive oxygen species in the *rns2*-2 mutant (Figure 5). Both *atg9*-4 and *rns2*-2*atg9*-4 had levels of reactive oxygen species similar to WT. Reactive oxygen species are known inducers of autophagy. Thus, it is possible that the increase in ROS observed in the *rns2*-2 mutant could be the signal that triggers the constitutive autophagy phenotype in these plants. To test this hypothesis, we blocked reactive oxygen species production using DPI treatment, which blocks ROS production by inhibition of NADPH oxidase, and imaged autophagy using MDC staining. When ROS production was inhibited through DPI treatment, *rns2*-2 lost its constitutive autophagy phenotype (Figure 6).

4.5 Discussion

Based on the number of differentially expressed genes and transcriptomic response, lack of fertilizer has a greater impact on gene expression than the genotypic differences under study (*rns2*-2, *atg9*-4 or *rns2*-2*atg9*-4). Further, the individual genotypes have different numbers and different differentially expressed genes when studied in either the nutritionally replete or nutritionally deplete condition, indicating a strong interaction between treatment and genotypes. This suggests that the mutations may be blocking different components of the ribophagy-like mechanism in plants, causing a different reaction to the loss of function of the gene in either the fertilized or unfertilized condition.
Comparison of our analysis of transcriptome changes in response to nutrient restriction to previous studies in nitrogen availability reveal a universal poor correlation between results. The largest similarity between these studies was approximately 12% between our transcriptome and Wang et al. Further, these analyses reveal that while key nitrate-oriented pathway enzymes, such as nitrate reductase, are not present in our list of differentially expressed genes, neither are they necessarily represented in all transcriptomes for nitrogen starvation, appearing in only one of the studied analyses. These differences may be due to differences in growing conditions, tissues or nitrogen source. Our statistically significant overlap was measured between differentially expressed genes in shoot samples from Wang et al and our own experiment [16]. Further analysis in differential expression in response to nitrogen limitation are necessary, as well as analysis of other fertilizer components including phosphate, potassium and micronutrients.

RNS2 and ATG9 are both required for maintenance of rRNA levels in the cell [6]. RNS2 is required for degradation of rRNA in the cell and, upon mutation, we observe disruption of the carbon flux pathways, particularly the pentose phosphate pathway. These changes in the pentose phosphate pathway translate into weakening of the cell wall and larger plant and cell size through reduction in the levels of cell wall components [9]. Several genes involved in sugar metabolism or transport were induced in our transcriptomic analysis. In the atg9-4 mutant, autophagic transport to the vacuole would be blocked, preventing recycling of key sugar-containing compounds, possibly resulting in a deficit of export of the key required nutrients from the plant vacuole. Efflux of sugars from the vacuole is very important, thus the plant retains multiple copies of efflux transporters, and our transcriptomic analysis showed that at least SWEET15 is impacted by defects in ATG9, indicating efflux must have some role which is disrupted in our autophagy mutants, either through loss of degradation or through maintenance of
homeostasis. Sugars such as galactose can cause reactive oxygen species signals in plants and are linked both to reactive oxygen species and antioxidant activity through oxidative cellular processes and through synthesis of antioxidant compounds such as anthocyanins [22]. Levels of reactive oxygen species in rns2-2 are higher than WT, indicative that rns2-2 exists in a state of high oxidative signals. Blockage of ROS production with DPI reveals that this increase in oxidative signals in rns2-2 may be the signal for constitutive autophagy.

Our results also further supported the posttranscriptional or posttranslational regulation of the ribophagy-like mechanism in Arabidopsis, consistent with posttranslational regulation of autophagy. Similarly to the previous microarray, rns2-2 under non-depleted conditions reveals relatively few differentially expressed genes, a surprising result in light of the disruption to homeostasis and the strong cellular phenotype. Regulation of autophagy by posttranslational mechanisms is well documented [23, 24], and strongly supports the hypothesis that regulation of autophagy in rns2-2 may be at the posttranslational level, for example through activation by protein phosphorylation. Our results reinforce the relationship between mutation of rns2-2 and carbon flux components and cell wall modifying proteins previously reported. Our results also provided novel information on the relationship between the ribophagy-like mechanism in Arabidopsis and reactive oxygen species, and other studies have linked oxidative stress with carbon flux metabolites and carbon starvation, as carbon starvation in excised corn root tips results in decreased oxygen consumption which is controlled by the level of ADP [25].

Taken together, our results highlight the importance of reactive oxygen species in the response to loss of function in the ribophagy-like pathway for degradation of rRNA in Arabidopsis. Transcriptome analysis highlighted the effects in carbon and cell wall-associated pathways, as well as pathogen response and response to reactive oxygen species. We further
showed the elevation of reactive oxygen species in *rns2*-2 as well as the responsibility of ROS for the constitutive autophagy phenotype in *rns2*-2. As the transcriptomic impact of mutation of *RNS2* results in so few differentially expressed genes, further work on the posttranscriptional and posttranslational regulation in response to mutation of the ribophagy-like mechanism in Arabidopsis is necessary.

### 4.6 Funding

This work was supported by a grant from the United States National Science Foundation [MCB-1051818] to G.C.M. and D.C.B.

### 4.7 Acknowledgement

Thank you to Dr. Michelle Graham from the USDA-ARS for assistance with RNA-seq methodologies and analysis.
4.8 References


4.9 Figures and Tables

**Figure 1 - Global patterns of up- and downregulated genes.** The heatmap was generated using heatmap2.0 in R. A unique list of statistically significant differentially expressed genes across all genotypes was generated and a heatmap of differential expression of each gene was created for each comparison. This heatmap shows similarities between each of the three ribophagy-like process mutants (*rns2-2, atg9-4* and *rns2-2atg9-4*). A cladogram of similarity based on gene expression was created for both genes (left) and genotype (top).
Figure 2- Comparison of differentially expressed genes to previous nitrogen deficiency studies. A) A heatmap was generated using three previous studies by Scheible et al, Vidal et al and Wang et al who previously published nitrogen-deficiency studies using heatmap2.0. The heatmap includes the unique list of differentially expressed genes from the previous three studies and the differentially expressed genes from our fertilization experiment (Nutrition). B) Venny2.0 was used to create a venn diagram comparing the differentially expressed genes between our Nutrition differentially expressed genes, Vidal et al and Wang et al.
Figure 3- Comparison of differentially expressed genes in genotype comparisons. A venn diagram synthesized through Venny2.0 comparing the list of unique differentially expressed genes between comparisons of the rns2-2, atg9-4, and rns2-2atg9-4 samples against WT reveals subsets of shared differentially expressed genes between each genotype.
Figure 4-Metabolite analysis of pentose phosphate pathway components. Proportions of pentose phosphate components sedoheptulose-7-phosphate (S7P), fructose-6-phosphate (F6P), glucose-6-phosphate (G6P), 6-phosphogluconolactonate (6PGA), and unresolved ribose-5-phosphate, xylose-5-phosphate and ribulose-5-phosphate (R5P/Xu5P/Ru5P) were measured in WT (black), rns2-2 (white), atg9-4 (striped), and rns2-2atg9-4 (checked). Statistical analysis was performed between each genotype and WT using Student’s t-test at a significance cutoff of p < 0.05. Four to six samples were measured per two to four growth sets for each genotype.
Figure 5-Measurement of Reactive Oxygen Species. Seven-day old seedlings were stained with DCFDA for reactive oxygen species. Fluorescence was measured using microscopy and statistical comparison between WT and each mutant genotype was performed using student’s t-test (P < 0.05). Forty-four to sixty total measurements per genotype were taken from five to six independent growth sets.
Figure 6- Inhibition of Reactive Oxygen Species production  a) Roots of WT or rns2-2 were treated with DPI (NADPH oxidase inhibitor) or DMSO and then stained with MDC and imaged using confocal microscopy. b) The number of autophagosomes per frame was quantified and analysis performed using Student’s t-test (p>0.05) in comparisons against each treatment and genotype.
Table T1 Summary of differentially expressed genes in comparison against WT

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Supplemental Files:

**Supplemental File S1** – List of differentially expressed genes and their statistics

**Supplemental File S2** – List of differentially expressed genes represented in more than one comparison and the logFC in each sample in which the gene is noted as being differentially expressed.

**Supplemental File S3** – List of overrepresented Gene Ontologies

**Supplemental Table ST1** – Summary of read counts

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CHAPTER 5
CONCLUSIONS

5.1 Conclusions

This dissertation presents my work on the degradation of ribosomal RNA in *Arabidopsis thaliana* and the involvement of autophagy and the RNase T2 family in this process. While there is some understanding of the degradation of nonfunctioning or aberrant ribosomal RNA [1-3], a previous gap in understanding the mechanism of turnover of normal ribosomal RNA needed to be addressed. Plants must be able to undergo this process as ribosomal RNA forms a massive pool of resources in the cell, including segregation of phosphate and nitrogen. Under conditions of stress such as starvation or in different stages of cellular and organismal development, the plants must be capable of remobilizing ribosomal RNA resources through degradation or recycling of ribosomal RNA. In this dissertation I addressed how ribosomal RNA is degraded in a plant ribophagy-like mechanism which depends on the function of RNase T2 activity.

A mechanism of ribosomal RNA degradation exists in yeast known as ribophagy. In starvation conditions, the yeast ribosomal RNA is degraded in a process dependent on the core autophagy machinery but which is selective for ribosomal RNA [4]. When the RNase T2 in Arabidopsis, RNS2, is mutated (*rns2*-2), the plant displays constitutive autophagy and a longer rRNA half-life [5]. We present that this constitutive autophagy, which was preliminarily described, is indeed an autophagy mechanism, verifying that the small MDC stained bodies are autophagosomes, as well as that the autophagosomes contain RNA.

Using vacuole extraction combined with qPCR and acid phosphatase measurements, we demonstrate that these RNA-containing autophagosomes deliver their RNA cargo to the vacuole
in a mechanism dependent on the autophagy gene ATG5 for degradation by RNS2. Further, both ATG5 and ATG9, as well as RNS2, are required for normal rRNA levels in the plant, as plants with mutation in these or any combination of the autophagy genes and RNS2 display higher levels of total RNA. These results support the existence of a ribophagy-like autophagy of Arabidopsis ribosomal RNA. This mechanism is dependent on the autophagy machinery and the RNase T2 family and is performed in the vacuole.

Having established a mechanism for degradation of ribosomal RNA in Arabidopsis, we next endeavored to answer the question of why rns2-2 has a constitutive autophagy phenotype and the consequences of the rns2-2 mutation on cellular metabolism. We performed two transcriptomic studies. The initial study we performed was a microarray-based experiment in rns2-2 and WT seedlings. I examined the list of differentially expressed genes and found that loss of RNS2 function results in overrepresentation of differentially expressed genes involved in the cell wall, particularly expansins, which are associated with cell growth, and the carbon flux pathway, with emphasis around the pentose phosphate pathway. I analyzed the cell wall components and discovered mannose and glucuronic acid are lower in rns2-2. Further, we know that the rns2-2 basal rosette is larger than WT. Upon further examination, I found that rns2-2 has longer roots and longer root cells. This complements reported increased length of root hair length seen in microscopy images of rns2-2 plants [6]. The rns2-2 plants also have a higher water content compared to the WT. These results support an effect of alterations in cell wall composition feeding into phenotypic differences, either through weakening of the cell wall or through increases osmolyte (RNA) content of the cell.

As our initial transcriptomic analysis was performed only on rns2-2 and we later had access to rns2-2atg9-4 and atg9-4, and as RNA-seq provides greater depth of coverage and
flexibility in analysis, we performed RNA-seq transcriptomic analysis on WT, *rns2-2*, *atg9-4*, and *rns2-2atg9-4*. This second transcriptomic analysis was performed on adult plants, where most RNA analysis, including the vacuolar and total RNA analyses, was performed, in contrast to the microarray experiment in which seedlings, the stage used for most microscopy studies, were analyzed. During this analysis we found, again, involvement of cell wall associated components, reinforcing the phenotypic results and the results of the initial microarray-based differentially expressed genes analysis. A new and striking result is the involvement of oxidative stress or reactive oxygen species. Oxidative stress is a trigger for autophagy, providing an explanation for the induction of constitutive autophagy in *rns2-2*. To further examine the relationship between constitutive autophagy and reactive oxygen species we blocked reactive oxygen species production and observed loss of constitutive autophagy in *rns2-2*. Additionally, measurement of WT and *rns2-2* reactive oxygen species reveal *rns2-2* is in a high ROS state. This supports reactive oxygen species as the signal for constitutive autophagy in *rns2-2*.

A very striking result from both transcriptomic analyses is that, while we see strong impacts on homeostasis and growth, we see relatively short lists of differentially expressed genes, particularly in *rns2-2* and *rns2-2atg9-4*. These results suggests a strong probability that there is significant posttranscriptional or posttranslational regulation involved. Future research must be used to address this result and analyze these regulation mechanisms.
5.2 Future Directions

Research remains to be conducted in this area. Chapter 2 has set the stage for the understanding of the selectiveness for rRNA degradation in plants. This can be studied using plant lines carrying mutations in candidate genes in selective autophagy. The Arabidopsis gene \textit{NEXT TO BRCA1 GENE1, NBR1} (AT4G24690) is an excellent candidate for further study. NBR1 is a gene in autophagy which interacts with ATG8 in the cargo identification process [7]. NBR1 acts as a cargo receptor for ubiquitinated proteins resulting in its selective degradation through autophagy [7, 8]. This interaction with ubiquitinated proteins is particularly interesting as ribophagy in yeast is dependent on the ubiquitin protease Ubp3 [4]. Additionally, while other cargo adaptors, such as p62, have been shown to mediate selective autophagy in animals and yet are not present in plants, NBR1 is present in both plants and animals and has specifically been shown to be degraded in the vacuole in plants as opposed to cargo adaptors in mammalian systems [9]. These data support the future study of NBR1 as a potential cargo adaptor in the ribophagy-like mechanism.

Further, whether ribosomal subunits are transported to the vacuole assembled or rRNA alone is transported into the vacuole needs to be addressed. These studies can be conducted using tagged rRNA and ribosomal proteins studying transport and degradation and through analysis of rRNA functionality in ribosomes extracted from vacuoles. However, an intriguing question remains in the ATG5-dependent, ATG9-independent rRNA degradation mechanism. Transport of rRNA through membrane proteins has been shown and several candidates exist in Arabidopsis [10].

One of the key areas yet to be studied is the mechanism of differential regulation of the carbon flux pathways. Flux analysis through the carbon flux pathways and \textit{de novo} nucleotide
synthesis will resolve whether the gene regulation and changes in metabolites in the carbon flux pathway are resulting in a shift of resources from the carbon flux pathway to de novo nucleotide synthesis.

Finally, it remains to be seen what is the relationship between \textit{rns2}-2 and reactive oxygen species. While we established there is an increase in bulk reactive oxygen species, we did not resolve the increase to a specific species. Depending on the source and concentration, reactive oxygen species may result in either oxidative stress or signaling. Oxidative stress is the result of overload of bulk peroxide resulting in stress to the cell. Plants are constantly producing reactive oxygen species through normal metabolic processes, and these species must be segregated and detoxified to prevent damage to proteins, lipids and nucleic acids. When these species accumulate in excess they produce oxidative stress which, under some conditions, signal programmed cell death [11]. Reactive oxygen species also serve in signaling in sub-stress levels and are a primary signal for autophagy [12]. Additionally, reactive oxygen species serve as a signal for stomatal closure which could further explain our high water content phenotype in \textit{rns2}-2 [13]. Through establishing the specific reactive oxygen species and their source, a greater understanding of how RNS2 loss impacts the plant can be established. Determination of the posttranslational impacts of \textit{rns2}-2 will help provide information both to this regulation of reactive oxygen species production and to the changes in carbon flux seen in \textit{rns2}-2 plants.

Taken together, the results presented in this dissertation reveal five key questions regarding the ribophagy-like process in plants which remain to be resolved. Is the autophagy mechanism that targets rRNA for degradation in Arabidopsis selective, and thus truly ribophagy-like? Is the rRNA being transported and degraded through the ribophagy-like mechanism as intact ribosomes, as ribosomal subunits, or as individual rRNAs? What is the mechanism of
ATG5-dependent, ATG9-independent rRNA autophagy? Is there flux from primary metabolism pathways to *de novo* nucleotide synthesis?

Answering these questions should provide a solid foundation for our understanding of rRNA turnover and its contributions to cellular metabolism that maintain plant homeostasis.

### 5.3 References


11. Dangl JL, Jones JD. Plant pathogens and integrated defence responses to infection. nature. 2001;411(6839):826-33.
