Peroxisome quality control: investigations of the roles of autophagy and the peroxisomal protease LON2

by

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Abstract

Peroxisomes are organelles that house numerous essential reactions producing reactive oxygen or nitrogen species as byproducts. Peroxisomes derive their name from hydrogen peroxide, which peroxisomes can detoxify using the enzyme catalase, but the hydrogen peroxide along with other reactive oxygen and nitrogen species can damage the resident peroxisomal enzymes.

Cells employ multiple mechanisms to maintain healthy populations of peroxisomes. In one peroxisome quality control mechanism, entire damaged or superfluous peroxisomes are degraded through autophagy, which is known as pexophagy when peroxisomes are selectively targeted. However, how healthy and damaged peroxisomes are distinguished is unknown. In another peroxisome quality control mechanism, the peroxisomal protease LON2 in plant cells is positioned to degrade or refold damaged proteins inside peroxisomes, but much about the function of LON2 is unknown, including its substrates.

To gain a better understanding of autophagy and LON2-mediated peroxisomal homeostasis, I took a variety of approaches using the reference plant *Arabidopsis thaliana*. I conducted a forward-genetic screen for *lon2* suppressors. Arabidopsis *lon2* mutants exhibit heightened pexophagy, resulting in several easily assayable phenotypes. I recovered 26 *lon2* suppressors disrupting several *AUTOPHAGY-RELATED (ATG)* genes: *ATG2*, *ATG3*, *ATG5*, *ATG7*, *ATG16*, and *ATG18a*. Three of these genes lack T-DNA insertional alleles in publicly available repositories, and the mutants that I recovered will be useful for future studies investing the functions of these genes. I also demonstrated that an insertional *atg11* allele only partially suppresses *lon2* defects and that the selective autophagy receptor NBR1 is neither necessary for *lon2* pexophagy nor sufficient for pexophagy in wild-type seedlings, indicating that Arabidopsis can utilize an NBR1-independent mechanism to target peroxisomes for degradation. I also characterized a series of *lon2* alleles and used site-directed mutagenesis to generate multiple *lon2* protein variants that will be useful in interrogating the functions of LON2. Finally, I developed a variety of fluorescent peroxisomal membrane reporters that will be useful for investigating pexophagy and other aspects of peroxisome biology in Arabidopsis. My research highlights the interconnection of autophagy and LON2 in peroxisome quality control and lays the groundwork for future studies elucidating this fascinating process.
Acknowledgements

None of this work would have been possible without the support and guidance of numerous individuals. The support and guidance that I received ranged from tangible, such as direct assistance with experiments, to intangible, such as encouragement on a bad day, and everything in between. My gratitude extends beyond what my words can effectively reach, but I would nonetheless like to acknowledge several of the individuals who provided guidance and support over the years.

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<tr>
<td>2,4-DB</td>
<td>2,4-dichlorophenoxybutyric acid</td>
</tr>
<tr>
<td>3-MA</td>
<td>3-methyladenine</td>
</tr>
<tr>
<td>AAA+</td>
<td>ATPase associated with various cellular activities</td>
</tr>
<tr>
<td>ACBP</td>
<td>Acyl-CoA binding protein</td>
</tr>
<tr>
<td>ABRC</td>
<td>Arabidopsis Biological Resource Center</td>
</tr>
<tr>
<td>ATG</td>
<td>Autophagy-related</td>
</tr>
<tr>
<td>ATI</td>
<td>ATG8-interacting</td>
</tr>
<tr>
<td>CaMV 35S</td>
<td>Cauliflower mosaic virus 35S promoter</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>DSK2</td>
<td>Dominant suppressor of KAR2</td>
</tr>
<tr>
<td>EMS</td>
<td>Ethyl methanesulfonate</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>F₁, F₂, etc.</td>
<td>Filial generation 1, 2, etc.</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GMT</td>
<td>GFP-mCherry-PEX26TM</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>IAA</td>
<td>Indole-3-acetic acid</td>
</tr>
<tr>
<td>IBA</td>
<td>Indole-3-butyric acid</td>
</tr>
<tr>
<td>ICL</td>
<td>Isocitrate lyase</td>
</tr>
<tr>
<td>M₁, M₂, etc.</td>
<td>Mutagenized plant generation 1, 2, etc.</td>
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<tr>
<td>Mito ATPase</td>
<td>Mitochondrial membrane complex V subunit α</td>
</tr>
<tr>
<td>MLS</td>
<td>Malate synthase</td>
</tr>
<tr>
<td>MT</td>
<td>mCherry-PEX26TM</td>
</tr>
<tr>
<td>NAA</td>
<td>1-naphtaleneacetic acid</td>
</tr>
<tr>
<td>NBR1</td>
<td>Neighbor of BRCA1 gene 1</td>
</tr>
<tr>
<td>PEX</td>
<td>Peroxin</td>
</tr>
<tr>
<td>PEX26TM</td>
<td>PEX26 transmembrane domain</td>
</tr>
<tr>
<td>PMP</td>
<td>Peroxisomal membrane protein</td>
</tr>
<tr>
<td>PMDH</td>
<td>Peroxisomal malate dehydrogenase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PTS</td>
<td>Peroxisome targeting signal</td>
</tr>
<tr>
<td>PXA1</td>
<td>Peroxisomal ABC Transporter 1</td>
</tr>
<tr>
<td>T₁, T₂, etc.</td>
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<tr>
<td>T-DNA</td>
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<tr>
<td>Ub</td>
<td>Ubiquitin</td>
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Chapter 1: Introduction

Parts of this chapter have been published (Young and Bartel, 2016) or are submitted for publication (Young et al., 2018).

1.1. Peroxisomes are essential organelles

1.1.1. Peroxisomes house numerous oxidative reactions

Peroxisomes are organelles that house a remarkable variety of oxidative processes essential for life. These organelles are present in virtually all eukaryotes. While some processes, such as β-oxidation of fatty acids and breakdown of hydrogen peroxide via catalase, are conserved across kingdoms, other processes are specialized. For example, mammalian peroxisomes are indispensable for synthesis of ether-phospholipids (reviewed in Wanders and Brites, 2010) whereas fungal peroxisomes host the last step of penicillin biosynthesis (Müller et al., 1991). Plant peroxisomes house various oxidative reactions including the glyoxylate cycle, several steps in photorespiration, and β-oxidation of fatty acids and hormone precursors (reviewed in Hu et al., 2012). Many peroxisomal enzymes produce hydrogen peroxide (H$_2$O$_2$) as a byproduct, and peroxisomes also house catalase and other enzymes that eliminate H$_2$O$_2$ (Fig. 1.1D). Unlike in mammals, where β-oxidation occurs in mitochondria as well as peroxisomes, peroxisomes are the sole site of β-oxidation in plants and fungi (reviewed in Graham, 2008). Plants with defective peroxisomes – and therefore impaired fatty acid β-oxidation – often require an exogenous fixed carbon source (e.g., sucrose) during seedling development when seed stores of triacylglycerol are normally catabolized (reviewed in Bartel et al., 2014a). Plant peroxisomes also use β-oxidation to convert the auxin precursor indole-3-butyric acid (IBA) to the active auxin indole-3-acetic acid (IAA) (reviewed in Strader and Bartel, 2011). The resultant IAA stimulates lateral root production in wild-type plants, and mutant plants with defective peroxisomes are often resistant to IBA because they inefficiently convert IBA into IAA (reviewed in Bartel et al., 2014a). I used lateral root production in response to IBA to query peroxisome functionality in my studies.
Figure 1.1 Working model of peroxisome biogenesis and metabolism.
Peroxisome biogenesis and protein import requires peroxins (PEX proteins), which are shown in colored ovals and indicated by numbers.

(A) Peroxisomes can be generated *de novo* from the ER. PEX3, PEX16, and PEX19 facilitate insertion of peroxisomal membrane proteins (PMPs) into membranes, which is necessary for budding of pre-peroxisomes from the ER.
(B) Peroxisome matrix proteins are imported using a suite of peroxins. Proteins bearing either a C-terminal peroxisomal-targeting sequence 1 (PTS1) or an N-terminal PTS2 bind to PEX5 or PEX7, respectively. The resulting complex docks with PEX13 and PEX14, allowing cargo import. Inside the peroxisome, the protease DEG15 removes the N-terminal PTS2 region of PTS2 proteins, and the protease LON2 is positioned to degrade obsolete or damaged matrix proteins (marked with a white asterisk).

(C) Following import, membrane-associated PEX5 is ubiquitinated via the PEX4 ubiquitin (Ub)-conjugating enzyme and the complex of Ub-protein ligases (PEX2, PEX10, and PEX12). Ubiquitinated PEX5 is removed from the peroxisomal membrane by a heterohexameric AAA-ATPase complex of PEX1 and PEX6, and de-ubiquitinated PEX5 can facilitate further rounds of import. Alternatively, polyubiquitinated PEX5 undergoes proteasomal degradation.

(D) Peroxisomes house several metabolic reactions. PXA1 imports fatty acids and the protoauxin indole-3-butyric acid (IBA), which undergo β-oxidation to generate acetyl-CoA and the auxin indole-3-acetic acid (IAA), respectively. The resulting acetyl-CoA is converted into succinate and malate, which contribute to seedling growth. The resulting IAA promotes lateral root development. Hydrogen peroxide (H₂O₂) is a byproduct of β-oxidation and is detoxified by the enzyme catalase (CAT).
1.1.2. Peroxins facilitate peroxisome biogenesis and import

Peroxin (PEX) proteins facilitate peroxisome biogenesis and protein import (Fig. 1.1; reviewed in Hu et al., 2012; Cross et al., 2016). Peroxisomal membrane proteins (PMPs) can be recognized by PEX19, a receptor that docks with PEX3 in the membrane to allow PMP insertion (Fig. 1.1A). Proteins delivered to the peroxisome matrix usually have one of two peroxisomal targeting signals: PTS1 or PTS2 (Fig. 1.1B). The PTS1 is a C-terminal, 3-amino-acid sequence recognized by PEX5; the PTS2 is a 9-amino-acid sequence near the N-terminus recognized by PEX7. In mammals and plants, PEX5 and PEX7 along with their respective cargos form a complex that docks with the PEX13 and PEX14 PMPs, allowing import of fully-folded proteins into the peroxisome matrix (reviewed in Hu et al., 2012). Once a PTS2 protein enters the peroxisome, the PTS2 region is removed to yield a mature form in plants (Helm et al., 2007; Schuhmann et al., 2008) and mammals (Swinkels et al., 1991). The resulting difference in molecular mass between precursor PTS2 proteins and mature proteins can be visualized by immunoblotting, providing a useful assay for characterizing plant peroxisome-defective mutants (reviewed in Bartel et al., 2014a; Kao et al., 2018), which often accumulate the precursor protein.

After cargo delivery, PEX5 in the peroxisomal membrane can be ubiquitinated through the action of peroxisomal ubiquitin-protein ligases (PEX2, PEX10, and PEX12) and PEX4, a peroxisome-tethered ubiquitin-conjugating enzyme (Fig. 1.1C). Ubiquitinated PEX5 is exported by a heterohexamer of PEX1 and PEX6 and can be degraded or de-ubiquitinated for use in further rounds of import (reviewed in Platta et al., 2014). The peroxins involved in PEX5 recycling are similar to the enzymes acting in endoplasmic reticulum (ER)-associated protein degradation (Schliebs et al., 2010; Bolte et al., 2011), and matrix proteins are stabilized in mutants defective in these peroxins (Zolman et al., 2005; Lingard et al., 2009; Burkhart et al., 2013; Burkhart et al., 2014).

1.1.3. Peroxisomes are essential for life in plants and humans

Autosomal recessive mutations in PEX genes confer lethal peroxisome biogenesis disorders in humans at a frequency of 1:50,000 in North America (reviewed in Braverman et al., 2016). Although null pex14 Arabidopsis thaliana mutants are viable (Monroe-Augustus et al., 2011), null mutants of most PEX genes confer lethality in A. thaliana (reviewed in Kao et al., 2018). While highlighting the importance of peroxins and peroxisomes for vitality, these data
also imply that quality control of peroxisomes must be maintained for prolonged viability. The wealth of genetic and biochemical tools available in *A. thaliana* make this plant a useful model for studying peroxisome biology, and the insights gained from studying Arabidopsis will likely be applicable to other eukaryotes.

1.2. Autophagy is an intracellular degradation pathway

1.2.1. Autophagy is a conserved process that degrades protein aggregates and organelles

Eukaryotes utilize macroautophagy, hereafter referred to as autophagy, to maintain cellular homeostasis during development and in response to environmental changes. Autophagy is a conserved cellular process used to degrade protein aggregates, pathogens, damaged or superfluous organelles, and other cellular components (reviewed in Mizushima and Levine, 2010; Li and Vierstra, 2012; Reggiori and Klionsky, 2013; Michaeli et al., 2016). During autophagy, a double membrane known as an isolation membrane (Fig. 1.2B, C) surrounds substrates to be degraded, forming an autophagosome (Fig. 1.2D, E). The autophagosome fuses with the lysosome (in animals) or vacuole (in plants and yeast) (Fig. 1.2F). Once in the vacuole, the complex, now referred to as an autophagic body, is lysed, the substrates are degraded, and the nutrients are exported to the cytosol for reuse (Fig. 1.2F).

1.2.2. Autophagy promotes plant survival during stress and starvation

Autophagy functions in stress responses, nutrient recycling, and protein quality control in plants. Because autophagy is not required for viability in *A. thaliana* (Doelling et al., 2002), null alleles lacking core autophagy components can be used to query the range of processes impacted by autophagy during plant growth and development.

Autophagy promotes plant survival during starvation and harsh environmental conditions and plays poorly understood roles in development. Autophagy is upregulated in plants during nutrient deprivation (Suttangkakul et al., 2011), and plants lacking autophagy are hypersensitive to carbon and nitrogen starvation (Doelling et al., 2002), supporting a role for autophagy in nutrient recycling. Plants lacking autophagy are also hypersensitive to fungal infection (Lai et al., 2011) and various abiotic stresses, including heat stress (Zhou et al., 2013), drought and salt
stress (Liu et al., 2009), and oxidative stress (Xiong et al., 2007), suggesting a role for autophagy in stress response. Autophagy is also upregulated during seed maturation (Di Berardino et al., 2018), suggesting a role during embryo development. Furthermore, plants lacking autophagy exhibit premature senescence, which is attributed to salicylic acid accumulation (Yoshimoto et al., 2009).

Autophagy also serves quality control functions in Arabidopsis. For instance, autophagy clears inactivated proteasomes (Marshall et al., 2015) and is induced following treatment with tunicamycin, which induces ER stress via the unfolded protein response (Liu et al., 2012). Although autophagy is not required for plant survival in optimal growth conditions (Doelling et al., 2002), plants lacking autophagy accumulate the selective autophagy receptor NBR1 (Svenning et al., 2011) and certain peroxisomal proteins (Kim et al., 2013; Shibata et al., 2013), suggesting that basal autophagy also functions in general intracellular housekeeping.

1.2.3. Core ATG genes are conserved

Over thirty AUTOPHAGY-RELATED (ATG) proteins have been identified, and roughly half of these proteins are part of the core autophagy machinery that is conserved across kingdoms, including the reference plant A. thaliana (reviewed in Li and Vierstra, 2012; Michaeli et al., 2016). These core ATG proteins can be divided into several groups. ATG1, ATG11, ATG13, and ATG101 are involved in initiating autophagosome formation (Fig. 1.2A; Suttangkakul et al., 2011; Li et al., 2014; Kang et al., 2018). ATG2, ATG6, ATG9, and ATG18 participate in isolation membrane expansion (Fig. 1.2B; Kihara et al., 2001; Xiong et al., 2005; Suzuki et al., 2007; Patel and Dinesh-Kumar, 2008; Wang et al., 2011; Zhuang et al., 2017; Kang et al., 2018). ATG8 is a ubiquitin-like protein that decorates the phagophore via conjugation to phosphatidylethanolamine (Fig. 1.2C; Ohsumi, 2001; Doelling et al., 2002; Sláviková et al., 2005). ATG3, ATG4, ATG5, ATG7, ATG10, ATG12, and ATG16 are needed to lipidate ATG8 (Fig. 1.2C; Doelling et al., 2002; Yoshimoto et al., 2004; Thompson et al., 2005; Phillips et al., 2008; Chung et al., 2010; Romanov et al., 2012; Yamaguchi et al., 2012). Much of our knowledge of the roles and mechanics of autophagy in plants comes from the study of mutants defective in ATG genes, which have predominantly been identified by homology-based comparison to ATG genes from yeast and studied using reverse genetics. In Chapter 3, I describe a facile forward-genetic screen to recover new autophagy-defective mutants in Arabidopsis.
Figure 1.2 Model of pexophagy in Arabidopsis thaliana.
A suite of AUTOPHAGY-RELATED (ATG) proteins enact autophagy and are essential for pexophagy. Colored ovals represent ATG genes that are characterized in Chapter 3. 

(A) ATG1, ATG11, ATG13, and ATG101 are involved in activation of isolation membrane assembly. ATG1, ATG13, and ATG101 are kinases whereas ATG11 acts as a scaffold for the complex.

(B) ATG2, ATG6, ATG9, and ATG18 are involved in isolation membrane expansion. ATG9 is a transmembrane protein that is speculated to bring lipids to the developing isolation membrane.

(C) ATG8 and ATG12 are ubiquitin-like proteins. ATG8 is first processed by the ATG4 protease to expose a C-terminal glycine. ATG7 is an E1-like protein that activates both ATG8 and ATG12 and coordinates transfer to their respective E2-like enzymes, ATG3 and ATG10. The ATG12–ATG5 conjugate associates with a dimer of ATG16, which facilitates tethering of the ATG12–ATG5 conjugate to the developing isolation membrane. The ATG12–ATG5-ATG16 complex acts as an E3-like enzyme for conjugating ATG8 to phophotidylethanolamine (gray bars), which is present in the developing isolation membrane.

(D) During pexophagy, condemned peroxisomes are selectively recruited to expanding isolation membranes. A selective autophagy receptor (brown ovals) is postulated to bind to the peroxisome and to ATG8, connecting the condemned organelle to the autophagy machinery. The signal on plant peroxisomes that is recognized by the selective autophagy receptor to mark the peroxisome for degradation has not been identified, but candidates include ubiquitinated proteins, such as PEX5 or a matrix protein; PMPs, such as PEX3 and PEX14; and oxidized or aggregated matrix proteins (marked with white X). ATG11 binds to the pexophagy receptor in yeast, but a similar role in Arabidopsis has not been established.

(E) The isolation membrane completely encloses its cargo, forming an autophagosome.

(F) The outer autophagosome membrane fuses with the vacuolar membrane, forming an autophagic body, and the contents of the autophagic body are degraded into their constitutive nutrients (e.g., amino acids and lipids) and released into the cytosol for reuse.
1.2.4. Substrates can be selectively targeted for autophagy

A wide variety of substrates can be degraded via autophagy, and the molecular mechanisms that enact various selective autophagy pathways continue to be unraveled. In addition to nonselective autophagy, which appears to promote survival under stress conditions, there is growing evidence for selective autophagy whereby specific proteins, protein complexes, infectious agents, or organelles are targeted for autophagy (reviewed in Li and Vierstra, 2012; Michaeli et al., 2016). Selective autophagy often requires receptors that link organelles, protein aggregates, or other cargo to the autophagy machinery by binding to both the fated cargo and ATG8 (Fig. 1.2D; reviewed in Rogov et al., 2014). These receptors generally bind ATG8 via an ATG8-interacting motif with the consensus core sequence W/Y/F-XX-L/I/V with neighboring acidic (D or E) residues (reviewed in Rogov et al., 2014; Xie et al., 2016). Receptors with differing specificity allow cells to selectively enact autophagy. Cells house many types of organelles, and the ability to recognize not only specific organelles but also damaged or unnecessary organelles is paramount to maintaining cellular homeostasis.

The list of selective autophagy receptors is growing (reviewed in Rogov et al., 2014; Michaeli et al., 2016). Two well-characterized mammalian receptors are Neighbor of BRCA1 gene 1 (NBR1) and sequestosome (SQSTM)-1 (also known as p62), which bind both ATG8 and ubiquitin (Rogov et al., 2014). Plants encode orthologs of NBR1 (known as Joka2 in tobacco) but not p62 (Svenning et al., 2011; Zientara-Rytter et al., 2011). Arabidopsis nbr1 mutants display sensitivity to only a subset of the stressors to which atg mutants display sensitivity (Zhou et al., 2013; Zhou et al., 2014), which is congruent with NBR1 acting as a selective autophagy receptor for a subset of cargo. Arabidopsis NBR1 is implicated in clearing ubiquitinated protein aggregates following heat stress (Zhou et al., 2013) and in limiting cauliflower mosaic virus infection by targeting virus particle-forming capsid proteins for degradation via autophagy (Hafrén et al., 2017). However, the specific types of selective autophagy directed by NBR1 are incompletely understood. Several ATG8-INTERACTING (ATI) plant-specific proteins (ATI1, ATI2, ATI3A, ATI3B, and ATI3C) emerged from screens for ATG8f-interacting proteins and associate with ER (Honig et al., 2012; Zhou et al., 2018). ATI1 plays a role in autophagy of plastids (Michaeli et al., 2014), and ATI3A is implicated in autophagy of ER (Zhou et al., 2018). Arabidopsis also uses the ubiquitin receptor DSK2 to target the transcription factor BES1 for degradation via autophagy (Nolan et al., 2017) and uses the RPN10 proteasome subunit as a
receptor for autophagy of proteasomes (Marshall et al., 2015). In Chapter 3, I explore the role of NBR1 in autophagy of Arabidopsis peroxisomes.

1.3. Pexophagy is the selective autophagy of peroxisomes

Pexophagy is specialized autophagy that degrades excess and damaged peroxisomes (Fig. 1.2D, E, F). Pexophagy is well-documented in yeast and mammals (reviewed in Till et al., 2012) and has long been postulated to exist in plants as well. A single electron micrograph of a peroxisome surrounded by a double membrane in a castor bean endosperm (Vigil, 1970) hinted at the occurrence of pexophagy in plants, but definitive evidence of pexophagy in plants only emerged very recently (Farmer et al., 2013; Kim et al., 2013; Shibata et al., 2013; Yoshimoto et al., 2014). However, the mechanisms by which plant cells recognize peroxisomes in need of turnover remain unclear.

1.3.1. Pexophagy is involved in quality control of plant peroxisomes

Pexophagy is important for peroxisome quality control in plants. Peroxisomal matrix proteins are subjected to oxidative damage from the $\text{H}_2\text{O}_2$ produced as a byproduct of $\beta$-oxidation, photorespiration, and other peroxisomal oxidative reactions (van den Bosch et al., 1992; Eastmond et al., 2000; Adham et al., 2005; Fahnenstich et al., 2008). Catalase detoxifies $\text{H}_2\text{O}_2$ by converting it to water and molecular oxygen (Willekens et al., 1997), but catalase is itself susceptible to damage from $\text{H}_2\text{O}_2$ (Anand et al., 2009). Autophagy-deficient seedlings accumulate peroxisomal aggregates of inactive catalase (Shibata et al., 2013; Yoshimoto et al., 2014), implying that pexophagy actively clears damaged peroxisomes. Furthermore, $\text{atg}2$ mutants exhibit clustered peroxisomes (Fig. 1.3A; Shibata et al., 2013), consistent with the possibility that the clustered peroxisomes are marked for degradation and gathered around the incompletely developed autophagy machinery that remains when ATG2 is absent.

Consistent with the notion that pexophagy mediates constant turnover of peroxisomes, pexophagy limits plant peroxisome abundance even in non-stress conditions. Moreover, pexophagy appears to occur at a higher basal rate than other types of selective autophagy, evidenced by the increased turnover of peroxisomal proteins relative to other organellar proteins. For example, leaves of $\text{atg}5$ mutants display increased levels of several peroxisomal proteins but wild-type levels of selected Golgi, ER, mitochondrial, and chloroplast proteins (Yoshimoto et al.,
2014), hinting that pexophagy may be more prevalent than autophagy of other organelles in seedling aerial tissue. Furthermore, Arabidopsis atg mutants have more peroxisomes than wild type (Fig. 1.3A; Kim et al., 2013; Shibata et al., 2013), and a maize atg mutant displays increased levels of the PEX14 PMP (Li et al., 2015). Moreover, both carbon-starved and rapidly dividing tobacco cells display increased peroxisome abundance when treated with an autophagy inhibitor (Voitsekhovskaja et al., 2014). These findings are all consistent with the possibility that pexophagy clears damaged peroxisomes at a basal rate in the absence of stresses typically associated with inducing autophagy.

Basal pexophagy rates appear to differ among various plant tissues. Arabidopsis atg mutants display increased peroxisome abundance and heightened peroxisomal protein levels in hypocotyls and leaves but not roots (Kim et al., 2013; Yoshimoto et al., 2014). A screen for mutants that stabilize a fluorescently labelled version of the peroxisomal enzyme isocitrate lyase (GFP-ICL) driven by the endogenous ICL promoter (Burkhart et al., 2013) recovered several atg7 and atg2 alleles, but these mutants show only weak and inconsistent stabilization of GFP-ICL in immunoblotting analysis of whole seedlings (Burkhart, 2013). The primary screen was performed by microscopic examination of hypocotyls of EMS-mutagenized M2 seedlings (Burkhart et al., 2013), suggesting that GFP-ICL stabilization might occur preferentially in hypocotyls. Indeed, ICL stabilization in atg5 and atg7 mutants is more apparent in extracts prepared from hypocotyls than in extracts prepared from entire seedlings (Kim et al., 2013).

1.3.2. Pexophagy is involved in peroxisome remodeling in maturing seedlings

Maturing seedlings shift the functional requirements of peroxisomes throughout maturation. Germinating oilseed plants, such as Arabidopsis, initially depend on fatty acid β-oxidation and the glyoxylate cycle to convert stored lipids into carbohydrates (reviewed in Graham, 2008). In addition to β-oxidation enzymes, early seedling peroxisomes, formerly called glyoxysomes (Pracharoenwattana and Smith, 2008), contain two glyoxylate cycle enzymes, isocitrate lyase (ICL) and malate synthase (MLS) (reviewed in Hu et al., 2012). The glyoxylate cycle allows the acetyl-CoA generated by fatty acid β-oxidation to be used to synthesize sugars. As seedlings mature and establish photosynthesis, the glyoxylate cycle becomes obsolete, and ICL and MLS are degraded (Zolman et al., 2005; Lingard et al., 2009). This developmental progression from seedling peroxisomes harboring glyoxylate cycle enzymes to leaf-type
peroxisomes harboring photorespiration enzymes provides model substrates with which to study peroxisome and peroxisome matrix protein degradation. Three basic models of peroxisome remodeling during early seedling development have been put forward: the two-population model, the one-population model, and the continuous turnover model (reviewed in Beevers, 1979).

In the two-population model, peroxisomes harboring glyoxylate cycle enzymes are degraded in the vacuole via autophagy, and peroxisomes housing photorespiration enzymes are synthesized de novo from the ER. However, autophagy mutants barely stabilize ICL and MLS (Farmer et al., 2013; Kim et al., 2013), failing to support a basic prediction of the two-population model.

In the one-population model, glyoxylate cycle enzymes exist together with photorespiration enzymes in the same peroxisomes, and a protease degrades ICL and MLS as seedlings transition to photosynthesis. Immunolabeling experiments using greening cucurbit cotyledons reveal both glyoxylate cycle and photorespiration enzymes in the same peroxisomes (Titus and Becker, 1985; Nishimura et al., 1986; Sautter, 1986), supporting the one-population model. Moreover, in vitro-synthesized MLS is stable when imported into peroxisomes purified from dark-grown seedlings or mature leaves but unstable when imported into transitional peroxisomes (Mori and Nishimura, 1989), suggesting that a peroxisomal protease is activated during the remodeling period. Reverse-genetic analyses of several Arabidopsis peroxisomal proteases failed to implicate a protease in this process (Lingard and Bartel, 2009); however, additional peroxisomal proteases (reviewed in van Wijk, 2015) remain to be tested. Rather than degradation by resident peroxisomal proteases, proteins might be retrotranslocated out of the peroxisome, polyubiquitinated, and degraded by the proteasome (Zolman et al., 2005; Lingard et al., 2009; Burkhart et al., 2013; Burkhart et al., 2014). However, this idea has proven difficult to definitively test because mutants defective in the PEX5-recycling peroxins implicated in ubiquitination and retrotranslocation (Fig. 1.1B) also have defects in matrix protein import (Burkhart et al., 2013; Burkhart et al., 2014), and efficient peroxisomal import is a prerequisite for efficient degradation of tested matrix proteins (Lingard et al., 2009; Burkhart et al., 2013).

In the continuous turnover model, peroxisomes are continuously formed de novo from the ER and degraded via autophagy. This model is based on the observation that peroxisomes associate with lipid bodies during germination, with lipid bodies and plastids during the transition period, and with plastids following lipid body depletion (Schopfer et al., 1976).
model received relatively little historical attention compared to the one- and two-population models but fits well with current findings.

### 1.3.3. Various pexophagy receptors have been identified in different systems

Several pexophagy receptors have been identified. *Pichia pastoris* Atg30 (Farré et al., 2008) and *Saccharomyces cerevisiae* Atg36 (Motley et al., 2012) act as pexophagy receptors, but plants lack Atg30 or Atg36 homologs. Mammalian NBR1 is necessary and sufficient for pexophagy and acts synergistically with p62, another selective autophagy receptor (Deosaran et al., 2013). As the only characterized pexophagy receptor from other organisms with a plant homolog (Svenning et al., 2011; Zientara-Rytter et al., 2011), NBR1 is an attractive plant pexophagy receptor candidate. Consistent with a role for NBR1 as a selective autophagy receptor, Arabidopsis * nbr1* mutants exhibit only a subset of *atg* mutant phenotypes. For example, both *nbr1* and *atg* mutants are susceptible to heat, drought, salt, and oxidative stresses and accumulate ubiquitinated substrates during heat stress (Zhou et al., 2013). However, *atg* mutants exhibit heightened age- and dark-induced senescence and fungal susceptibility, but *nbr1* mutants do not (Zhou et al., 2013). Peroxisomes are likely essential for fatty acid β-oxidation during age-and dark-induced senescence (Dong et al., 2009), and some of the oxidative reactions in peroxisomes, including jasmonate biosynthesis, are critical for resistance to fungal infection (Rowe et al., 2010), suggesting that peroxisomes may need to avoid pexophagy during certain stresses that increase general autophagy, which is postulated to preferentially break down substrates not essential for survival during stress. Intriguingly, quantitative proteomic analysis identified several peroxisomal matrix proteins among the proteins that over-accumulate in *nbr1* mutants during heat stress (Zhou et al., 2014), consistent with the possibility that NBR1 promotes pexophagy during heat stress. However, no direct evidence has connected NBR1 to pexophagy in plants. In Chapter 3, I describe my experiments investigating the role of NBR1 in pexophagy, which demonstrated that NBR1 is neither necessary for *lon2*-related pexophagy nor sufficient to induce pexophagy in wild-type seedlings. Considering the variety of pexophagy receptors in different organisms, it would not be surprising if plants have novel pexophagy receptors.
1.3.4. Numerous peroxisomal membrane proteins have been implicated in pexophagy

A signal on the surface of the peroxisome is postulated to mark peroxisomes for pexophagy. Several peroxins reside in the peroxisome membrane and have been implicated in pexophagy in Arabidopsis as well as yeast and mammalian cells. In general, peroxins or other peroxisomal membrane proteins might mediate pexophagy in three ways.

First, post-translationally modified peroxins could interact with a selective autophagy receptor. In mammalian cells, PEX5 phosphorylation by ataxia-telangiectasia mutated (ATM) leads to pexophagy (Zhang et al., 2015). Selective autophagy receptors, including NBR1, often recognize ubiquitinated cargo (reviewed in Rogov et al., 2014; Michaeli et al., 2016). Because the matrix protein import peroxin PEX5 is ubiquitinated during its typical function (Fig. 1.1C; Platta et al., 2009), ubiquitinated PEX5 is a candidate pexophagy signal. Overexpression of PEX3 in mammalian cells induces ubiquitin-linked pexophagy, but PEX3 is not ubiquitinated in this process (Yamashita et al., 2014). Moreover, expression of ubiquitin fused to the transmembrane domain of a PMP is sufficient to induce pexophagy in mammalian cells (Kim et al., 2008), which is consistent with the possibility that a ubiquitinated PMP ordinarily serves as a pexophagy signal. I describe my attempts to test the sufficiency of ubiquitin for Arabidopsis pexophagy in Chapter 5.

Second, peroxisomal membrane proteins could interact directly with a selective autophagy receptor. For example, *Pichia pastoris* Atg37 (known as Acyl-CoA Binding Protein 5 in mammals) is a peroxisomal membrane protein that binds to the pexophagy receptor Atg30 and modulates pexophagy (Nazarko et al., 2014; Zientara-Rytter et al., 2017). Moreover, the membrane peroxins Pex3 and Pex14 interact with the pexophagy receptors Atg30 in *P. pastoris* (Farré et al., 2008; Burnett et al., 2015; Zientara-Rytter et al., 2017) and NBR1 in mammalian cells (Jiang et al., 2015). Pex3 also binds to the *Saccharomyces cerevisiae* pexophagy receptor Atg36 (Motley et al., 2012), and PEX3 plays a pivotal role in pexophagy in mammalian cells (Yamashita et al., 2014). Decreasing PEX14 levels in mammalian cells reduces pexophagy, perhaps as a result of reduced recruitment of PEX5 (Deosaran et al., 2013). The membrane peroxins PEX2 and PEX12 interact with Arabidopsis DSK2 (Kaur et al., 2013), which also serve as a selective autophagy receptor (Nolan et al., 2017), making these peroxins attractive candidates for pexophagy regulators.
Third, peroxins could interact directly with ATG8, bypassing the need for a bridging receptor. For instance, PEX14 directly binds LC3-II (the mammalian homolog of lipidated ATG8) (Jiang et al., 2015). Although the relative simplicity of ATG8-interacting motifs has confounded bioinformatic approaches to identifying ATG8-interacting proteins, a recent refinement considering acidic residue placement revealed that Arabidopsis PEX10 and PEX6 interact with ATG8 (Xie et al., 2016). However, pexophagy contributes to defects observed in \textit{pex1} and \textit{pex6} mutants in Arabidopsis (Rinaldi et al., 2017; Gonzalez et al., 2018), yeast (Nuttall et al., 2014), and humans (Law et al., 2017), suggesting that these peroxins prevent rather than promote pexophagy, perhaps by competing with the pexophagy signal for interaction with ATG8 or by removing ubiquitinated substrates, such as PEX5, from the peroxisomal surface.

### 1.3.5. Tools for monitoring autophagy and pexophagy in plants

Several methods are available to monitor autophagy in Arabidopsis. The selective autophagy receptor NBR1 accumulates when autophagy is prevented (Svenning et al., 2011), and I monitored NBR1 levels to identify autophagy-defective mutants in Chapter 3 and to assess the autophagy-inhibiting efficacy of various chemicals in Chapter 4. ATG8 fused to a fluorescent protein such as green fluorescent protein (GFP-ATG8) serves as an autophagosome marker (Thompson et al., 2005). In Chapter 4, I examined the localization of GFP-ATG8a in a mutant with heightened autophagy of peroxisomes. Autophagic flux can be monitored using a tandem-fluorescent protein comprised of GFP and mCherry (Pankiv et al., 2007). GFP and mCherry have different pKa values (~6.0 and 4.5, respectively) and only fluoresce at pH values above their respective pKa (Shaner et al., 2005). The vacuole has a pH of ~4.7 (Shen et al., 2013), so vacuolar GFP is quenched while mCherry continues to fluoresce (Pankiv et al., 2007). This tandem-fluorescent protein can be fused to the transmembrane domain of a peroxisomal membrane protein to monitor the rate of autophagy of peroxisomes (Deosaran et al., 2013). Using confocal microscopy, puncta with co-localized GFP and mCherry correspond to cytosolic peroxisomes; mCherry puncta that do not co-localize with GFP correspond to vacuolar peroxisomes that have undergone pexophagy (Deosaran et al., 2013). In Chapter 5, I describe a tandem-fluorescent construct that I developed for monitoring autophagy of Arabidopsis peroxisomes.
1.4. LON2 is a peroxisomal protease involved in peroxisome quality control

In addition to peroxisome quality control by pexophagy, the LON2 peroxisomal protease helps maintain peroxisome quality control. As a peroxisomal protease, LON2 is positioned to degrade glyoxylate cycle enzymes. However, lon2 mutants fail to stabilize ICL or MLS (Lingard and Bartel, 2009; Burkhart et al., 2013). A suppressor screen revealed that disabling autophagy genes in lon2 mutants results in dramatic ICL and MLS stabilization, indicating that autophagy is involved in degrading peroxisomal proteins when LON2 is nonfunctional (Farmer et al., 2013; Bartel et al., 2014b) and that both autophagy and the LON2 protease are involved in maintaining peroxisome homeostasis. In Chapter 3, I describe an optimized screen for lon2 suppressors to identify ATG genes in Arabidopsis that recovered more than 20 novel alleles in six different ATG genes. In Chapter 4, I characterize a series of lon2 alleles that originated from various forward-genetic screens conducted in the Bartel lab that I characterized to better understand the role of the LON2 protease in peroxisome quality control.

1.4.1. LON2 prevents pexophagy

LON2 plays an important but ill-defined role in preventing pexophagy. A critical role for LON2 in peroxisome physiology is indicated by the peroxisome-defective phenotypes exhibited by lon2 mutants: enlarged peroxisomes (Fig. 1.3A), IBA resistance (inefficient β-oxidation of IBA into IAA; Fig. 1.3B), PTS2-processing defects (Fig. 1.3C), and reduced matrix protein import (Fig. 1.3A, C; Lingard and Bartel, 2009; Farmer et al., 2013; Goto-Yamada et al., 2014). Interestingly, these defects worsen as cells mature (Lingard and Bartel, 2009), consistent with the possibility that lon2 peroxisomes are functional in recently divided cells (e.g., in young cotyledons and root tips) but are degraded by pexophagy at an increased rate compared to wild type as cells mature. A lon2 suppressor screen revealed that preventing autophagy restores all four of the peroxisome-related phenotypes observed in lon2 mutants (Fig. 1.3; Farmer et al., 2013), indicating that lon2 phenotypes are a result of heightened pexophagy. Notably, ICL and MLS are degraded at wild-type rates in lon2 mutants but are stabilized when both LON2 and autophagy are disrupted (Fig. 1.3C; Farmer et al., 2013; Goto-Yamada et al., 2014), demonstrating roles for both pathways in plant peroxisome homeostasis. In contrast, the peroxisomal enzyme thiolase is destabilized in lon2 mutants (Lingard and Bartel, 2009) but
Figure 1.3 Preventing autophagy suppresses phenotypes in Arabidopsis seedlings lacking a functional copy of the peroxisomal protease LON2.

(A) Preventing autophagy suppresses protein import defects and the large puncta phenotype of lon2-2. Cotyledon mesophyll cells in 8-d-old seedlings of the indicated genotypes expressing 35S:PTS2-GFP were imaged using confocal microscopy. PTS2-GFP bears an N-terminal PTS2 that localizes GFP (green) to peroxisomes in wild-type Columbia-0 (Wt), atg2-4 and lon2-2 atg2-3. PTS2-GFP is partially cytosolic (diffuse fluorescence) in lon2-2. Peroxisomes are enlarged in lon2-2 and clustered in atg2-4 and lon2-2 atg2-3. Chlorophyll autofluorescence (magenta) marks chloroplasts. Scale bar = 10 µm.

(B) Preventing autophagy suppresses the IBA resistance of lon2-2. Wt, lon2-2, atg2-4, and lon2-2 atg2-3 seedlings were plated on medium containing 0.5% sucrose (mock) and grown for 4 days. Half of the seedlings were then transferred to medium supplemented with 0.5% sucrose and 10 µM IBA, and seedlings were grown for an additional 4 days before measuring lateral root formation. Error bars represent standard deviation of the means (n ≥ 10).

(C) Preventing autophagy suppresses the PTS2-processing defect of lon2-2, and obsolete matrix proteins are stabilized in lon2-2 atg2-3 double mutants. Extracts from 6-day-old light-grown seedlings of the indicated genotypes were processed for immunoblotting in duplicate, and membranes were serially probed with antibodies recognizing the peroxisome matrix proteins thiolase or malate dehydrogenase (PMDH), shown in the upper panel, or malate synthase (MLS), shown in the lower panel. Thiolase and PMDH are PTS2 proteins synthesized as precursors (p) in the cytosol and cleaved to mature forms (m) lacking the PTS2 region in the peroxisome. Protein loading was monitored by probing with an antibody recognizing HSC70.

(Figure modified from Young and Bartel, 2016.)
stabilized when autophagy or both LON2 and autophagy are disrupted (Fig. 1.3C; Farmer et al., 2013; Goto-Yamada et al., 2014), hinting that thiolase is primarily turned over via pexophagy.

Intriguingly, a mitochondrial Lon isoform regulates mitophagy (selective autophagy of mitochondria) in Drosophila (Thomas et al., 2014). The serine/threonine kinase PINK1 (PTEN-induced putative kinase 1) and the ubiquitin-protein ligase Parkin are central to mitophagy. Depolarized mitochondria accumulate PINK1 on the outer membrane, where it phosphorylates Ser65 of ubiquitin, which activates Parkin to ubiquitinate outer mitochondrial membrane proteins (Narendra et al., 2010; Vives-Bauza et al., 2010; Kane et al., 2014; Kazlauskaite et al., 2014; Koyano et al., 2014). This ubiquitination provides additional PINK1 substrates, forming a positive feedback loop, and also marks the damaged mitochondria for degradation via mitophagy (Narendra et al., 2008). In Drosophila, healthy mitochondria avoid mitophagy by constitutively degrading PINK1, and several proteases have been implicated in this degradation, including mitochondrial Lon (Thomas et al., 2014). When mitochondrial membrane potential is disrupted, these proteases are rendered nonfunctional, allowing PINK1 to accumulate and trigger mitophagy (Jin et al., 2010; Thomas et al., 2014). Although the Arabidopsis mitochondrial isoforms LON1 and LON4 have not been directly implicated in mitophagy, lon1 mutants display enlarged mitochondria (Rigas et al., 2009), reminiscent of the large peroxisomes in lon2 mutants (Fig. 1.3A; Farmer et al., 2013; Goto-Yamada et al., 2014). It is tempting to speculate that Arabidopsis LON2 plays a role in plant peroxisomes analogous to the role that Drosophila Lon plays in mitochondria by degrading or disaggregating pexophagy-promoting factors in peroxisomes.

LON2 might prevent pexophagy by degrading oxidized peroxisomal proteins, and increased oxidative damage could explain the prevalence of pexophagy in aerial tissue. The peroxisomal enzyme glycolate oxidase, which converts glycolate into glyoxylate during photorespiration, generates H$_2$O$_2$ as a byproduct (Fahnenstich et al., 2008), and photorespiration occurs in aerial tissue but not in roots. Intriguingly, elevated CO$_2$ suppresses the enlarged peroxisome phenotype of lon2 mutants (Goto-Yamada et al., 2014). This suppression is presumably due to decreased photorespiration (and in turn reduced H$_2$O$_2$ production), consistent with a role for photorespiration in promoting oxidative damage of peroxisomes. It was not reported whether this CO$_2$-mediated suppression is light-dependent, which would be expected if photorespiration is required for the large-peroxisome phenotype observed in lon2 leaves.
Together, available data suggest that increased oxidative damage necessitates more peroxisome turnover via pexophagy, particularly in aerial tissues.

1.4.2. LON proteins are AAA+ proteases

LON proteins are a conserved family of homo-oligomeric ATPases with both chaperone and protease activities that are involved in protein quality control (reviewed in Iyer et al., 2004; Venkatesh et al., 2012). LON proteases form hexameric complexes with a central pore through which substrates are thought to be threaded (reviewed in Gur, 2013). LON monomers are typically composed of an N-terminal domain that is involved in recognizing substrates and maintaining the overall structure of the hexamer (Wohlever et al., 2014; Kereïche et al., 2016), a central AAA (ATPase Associated with diverse cellular Activities) domain containing canonical Walker A and Walker B motifs as well as an arginine finger, and a C-terminal protease domain containing a Ser-Lys catalytic dyad (reviewed in Venkatesh et al., 2012; Rigas et al., 2014).

Eukaryotic LON isoforms are localized to organelles. In Arabidopsis, LON isoforms are targeted to mitochondria, chloroplasts, and peroxisomes (van Wijk, 2015). LON1 (Daras et al., 2014) and LON4 (Ostersetzer et al., 2007) are dually localized to mitochondria and chloroplasts, and LON3 appears to be a pseudogene (Ostersetzer et al., 2007). LON2 contains a canonical PTS1 and is localized to peroxisomes (Ostersetzer et al., 2007; Reumann et al., 2009; Goto-Yamada et al., 2014).

1.4.3. LON proteases function as both chaperones and proteases

Like E. coli Lon (Gur and Sauer, 2008; Wohlever et al., 2014) and yeast peroxisomal Lon (Aksam et al., 2007; Bartoszewska et al., 2012), LON2 is an AAA ATPase thought to act as both a chaperone and a protease that recovers misfolded proteins and degrades proteins that cannot be refolded (Goto-Yamada et al., 2014). Expressing variants of LON2 with an inactive AAA domain or an inactive protease domain reveals distinct roles for LON2 domains in peroxisome maintenance (Goto-Yamada et al., 2014). Expressing protease-deficient, AAA-active lon2 in a lon2 null mutant prevents the rampant pexophagy that typifies lon2 mutants (Goto-Yamada et al., 2014), suggesting that the AAA domain of LON2 normally prevents excessive pexophagy, perhaps by acting as a chaperone. Indeed, expressing AAA-deficient, protease-active lon2 in a lon2 null mutant fails to prevent excess pexophagy (Goto-Yamada et al., 2014). Intriguingly,
glyoxylate cycle enzymes are stabilized when the protease-deficient, AAA-active lon2 is expressed in a lon2 mutant (Goto-Yamada et al., 2014), implicating the protease domain in matrix protein turnover. Moreover, peroxisomes in lon2 mutants expressing protease-deficient lon2 are both clustered and more abundant than in wild type, resembling peroxisomes in an atg2 mutant (Goto-Yamada et al., 2014), suggesting that impeding the protease domain of LON2 inhibits pexophagy. Thus it appears that the AAA domain of LON2 restraints pexophagy regardless of the activity of the protease domain, and the protease activity of LON2 promotes pexophagy but only when the AAA domain is present (Goto-Yamada et al., 2014). In Chapter 4, I describe site-directed mutagenesis experiments to generate AAA-inactive, protease inactive, and other lon2 mutants to further explore the roles of LON2 as a chaperone and a protease.

1.4.4. LON proteases recognize a broad set of substrates

A LON2 substrate likely accumulates in lon2 mutants and stimulates pexophagy. However, the molecules that target peroxisomes for pexophagy in lon2 mutants remain unidentified. Immuno-electron microscopy analysis of a lon2 mutant reveals enlarged, irregularly-shaped peroxisomes harboring electron-dense regions containing nonfunctional catalase (Goto-Yamada et al., 2014), suggesting that aggregated catalase might trigger pexophagy in lon2 mutants. Similarly, peroxisomes in the fungus Penicillium chrysogenum lacking peroxisomal Lon accumulate nonfunctional catalase-peroxidase (Bartoszewska et al., 2012). Moreover, mitochondria in S. cerevisiae lacking mitochondrial Lon accumulate electron-dense inclusions (Suzuki et al., 1994). Together, these data demonstrate the importance of LON proteases in maintaining quality of proteins inside their respective organelles. However, although the fate of peroxisomes in plants lacking both LON2 and catalase has not been reported, catalase is not necessary for the peroxisome clustering that is observed in atg2 mutants (Shibata et al., 2013), suggesting that catalase aggregates might be a symptom of peroxisome dysfunction rather than a signal for pexophagy.

LON proteases recognize a broad set of substrates but act with high selectivity. In other words, to effectively regulate quality control, LON proteases must be able to degrade a large variety of proteins but must only degrade these proteins under certain circumstances such as damage or aggregation. Several substrates, such as SulA and β-galactosidase, have been well established for E. coli Lon (Gur and Sauer, 2008; Wohlever et al., 2014), but no consensus
sequence is apparent among these substrates, making \textit{a priori} identification of LON substrates difficult. Degradation tags are typically rich in hydrophobic residues and are buried in natively folded proteins but exposed in misfolded proteins (Gur and Sauer, 2008), which may allow LON to selectively recognize and degrade misfolded proteins. More directed approaches will be needed to identify and validate substrates on LON2 in plant peroxisomes, and in Chapter 4, I describe site-directed mutagenesis to develop a lon2 variant that is expected to act as a substrate trap, which may allow identification of LON2 substrates. I also developed other lon2 variants that will help to decipher the role of LON2 in maintaining peroxisome quality control.

1.5. Overview of this thesis

In Chapter 2, I describe the details on the materials and methods used in this thesis. In Chapter 3, I describe a forward-genetic screen for lon2 suppressors, which recovered more than 20 unique \textit{atg} alleles in six different genes. I also describe experiments demonstrating that an \textit{atg11} mutant only partially suppresses lon2 and that NBR1 is neither necessary for lon2 pexophagy nor sufficient to induce pexophagy in wild type. In Chapter 4, I discuss a lon2 allelic series that I characterized, investigate the large puncta phenotype in lon2, and explore the efficacy of various chemicals to suppress lon2 defects. I also detail my site-directed mutagenesis designed to probe LON2 functions. In Chapter 5, I describe a tandem-fluorescent reporter that I generated for monitoring pexophagy in Arabidopsis. Finally, in Chapter 6, I consider how this work impacts the current thinking of peroxisome quality control and possible future studies that could provide insight into this fascinating process.
Chapter 2: Materials and methods

Parts of this chapter are submitted for publication (Young et al., 2018).

2.1. Plant material and growth conditions

2.1.1. Arabidopsis accession and mutants

The Columbia-0 (Col-0) accession of Arabidopsis thaliana was used as the wild-type (wt) control for the experiments in Chapters 4 and 5 and in Fig. 3.10 and as the second wild-type control in the Fig. 3.2C immunoblot, and Col-0 expressing 35S:GFP-PTS1 (Zolman and Bartel, 2004) was used as the wild-type control for the remaining experiments in Chapter 3. lon2-2 (SALK_043857) (Lingard and Bartel, 2009) crossed to 35S:GFP-PTS1 was mutagenized to isolate suppressors (see 2.1.4. below) and used as the lon2-2 control for subsequent experiments in Chapter 3. lon2-2 atg2-4 35S:PTS2-GFP and lon2-2 atg7-4 35S:PTS2-GFP were previously described (Farmer et al., 2013). atg7-3 (SAIL_11_H07) (Lai et al., 2011; Wang et al., 2011), atg11-1 (SAIL_1166_G10) (Li et al., 2014), nbr1-1 (SALK_135515) (Zhou et al., 2013), and nbr1-2 (GABI_246H08) (Zhou et al., 2013) were previously described and were obtained from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University. lon2-2 atg7-9 35S:PTS2-GFP is an unpublished allele originating from a screen for lon2-2 35S:PTS2-GFP suppressors (Farmer et al., 2013). atg2-4 35S:PTS2-GFP, atg3-1 35S:PTS2-GFP, and atg7-4 35S:PTS2-GFP were obtained by crossing the respective lon2-2 atg 35S:PTS2-GFP double mutant (Farmer et al., 2013) to 35S:PTS2-GFP (Woodward and Bartel, 2005) to remove the lon2-2 mutation. The presence of the T-DNA insertion in atg7-3, atg11-1, lon2-2, lon2-3 (GABI_034C09), nbr1-1, and nbr1-2 was confirmed by genomic PCR of using a gene-specific primer and a T-DNA left-border specific primer (Table 2.1), and progeny of homozygous lines of the respective mutants were used.

nbr1-4 (WiscDsLoxHs007_07A) was obtained from the ABRC. The position of the T-DNA insertion was confirmed by genomic PCR of using a gene-specific primer and a T-DNA left-border specific primer (Table 2.1), and the exact position of the insertion was determined by sequencing the PCR product (Lone Star Labs, Houston, TX).
Table 2.1 Genotyping primers for detecting T-DNA insertional mutations

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<tr>
<th>Allele</th>
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<tr>
<td></td>
<td>ATG7-Tsp45I</td>
<td>GTCGATTTAAAAATTTAATGAGGTG</td>
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<td>atg7-3</td>
<td>LB1-SAIL</td>
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<td>~350</td>
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<td>ATG11-1</td>
<td>AAGATGCTAGTTACATTTCCCGTTTTT</td>
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<td></td>
<td>ATG11-2</td>
<td>TCGATATGTAACCAACATAGAATCAAGG</td>
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<tr>
<td>atg11-1</td>
<td>LB1-SAIL</td>
<td>GCCCTTTAGAAATGGATAATAGCCTTGCTTC</td>
<td>~300</td>
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<tr>
<td></td>
<td>LON2-17</td>
<td>TGGATTCCTTACCATATGGGCCACAGTCC</td>
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</tr>
<tr>
<td>lon2-2</td>
<td>LB1-SALK</td>
<td>CAAACCAGCTGAGCTGCTGCAACTCC</td>
<td>~350</td>
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<tr>
<td>LON2</td>
<td>LON2-20</td>
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<tr>
<td></td>
<td>LON2-21</td>
<td>CTCCCGAAGTTCTTCATAGCATACAAGC</td>
<td></td>
</tr>
<tr>
<td>lon2-3</td>
<td>GABI-3144</td>
<td>GTGGATGATGATGATATCTCCC</td>
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<tr>
<td></td>
<td>LON2-21</td>
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</tr>
<tr>
<td>NBR1</td>
<td>NBR1-2</td>
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<td>380</td>
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<tr>
<td></td>
<td>NBR1-3</td>
<td>TTCTTCTGTATTTCTATGTGCTTCTCTG</td>
<td></td>
</tr>
<tr>
<td>nbr1-1</td>
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<td>NBR1</td>
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<td>NBR1-13</td>
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<td>NBR1</td>
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<td>370</td>
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<td></td>
<td>NBR1-7</td>
<td>CTCCATTGTTCTCTTTCTAGCACTTC</td>
<td></td>
</tr>
<tr>
<td>nbr1-4</td>
<td>WiscDsLoxHs L4</td>
<td>TGATCCATTGAGATTCCCCGAGATGAAG</td>
<td>~300</td>
</tr>
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</table>
lon2-4 (Farmer et al., 2013), lon2-6 (Burkhart et al., 2013), lon2-8, lon2-9, lon2-10 (Rinaldi et al., 2016), lon2-277, and lon2-310 (Fleming, 2016) were previously described. lon2-5 (alias SDI154) is an unpublished allele recovered from a screen for sucrose dependence and IBA resistance (Woodward and Bartel, unpublished). PCR-based genotyping markers designed using dCAPS Finder 2.0 (Neff et al., 2002) were used to confirm the presence of the identified lesions (Table 2.2).

### 2.1.2. lon2-2 suppressor physiological characterization

Seeds were surface-sterilized using 3% (v/v) bleach solution containing 0.01% (v/v) Triton X-100, resuspended in 0.1% agar, and stratified at 4 °C overnight or up to 3 days. Seedlings were grown at 22 °C on plant nutrient (PN) medium (Haughn and Somerville, 1986) supplemented with 0.5% (w/v) sucrose (PNS) and solidified with 0.6% (w/v) agar. To quantify IBA sensitivity of lateral root production, seedlings were grown on PNS plates under continuous white light for 4 days, transferred to new PNS plates with either ethanol (mock) or 10 µM IBA (from a 100-mM stock in ethanol), and grown under light filtered with yellow long-pass filters (to slow photochemical breakdown of indolic compounds; Stasinopoulos and Hangarter, 1990) for an additional 4 days. Lateral roots emerged from the primary root were counted using a dissecting microscope, and primary root lengths were measured using a ruler. Measurements were completed at least twice with similar results.

### 2.1.3. Chemical treatment of lon2-2

To test the ability of various chemicals to suppress lon2-2 defects, seeds were surface-sterilized and stratified as described above. Seedlings were grown in 12-well plates at 22 °C on 2 mL of PNS with the desired concentration of DMSO or the chemical of interest and with or without IBA or 2,4-DB. For root measurements, seedlings were grown for 6 or 8 days as indicated under light filtered with yellow long-pass filters. Lateral roots emerged from the primary root were counted using a dissecting microscope, and primary root lengths were measured using a ruler. For dark-grown-hypocotyl measurements, seeds were placed at 22 °C under light filtered with yellow long-pass filters to stimulate germination then wrapped in aluminum foil and grown at 22 °C for an additional 5 days. Hypocotyls lengths were measured using a ruler.
<table>
<thead>
<tr>
<th>Mutant</th>
<th>Primer name</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Restriction Enzyme</th>
<th>Fragment size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>lon2-4</td>
<td>LON2-DdeI</td>
<td>AGCTATGTTTTTTTTTTTTTTCTAATACTCA</td>
<td>DdeI</td>
<td>89, 69, 115, 69, 26</td>
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<tr>
<td></td>
<td>LF1043</td>
<td>CGAGCGATGTAATCCGAGCAA</td>
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<tr>
<td>lon2-5</td>
<td>LON2-2s</td>
<td>CCACATCTTTTCTTCTTCTGCTGG</td>
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</tr>
<tr>
<td></td>
<td>LON2-3s</td>
<td>CTTATCTTTTGATGCCACCAACAGGCAGA</td>
<td>HpaII</td>
<td>120, 55, 175</td>
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<td>lon2-6</td>
<td>LON2-26</td>
<td>AATTTGTTGCTTTTCTTCTTGTGGTGT</td>
<td>Mnll</td>
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<td>LON2-21</td>
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<td>LON2-30</td>
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<td>57, 42, 99</td>
</tr>
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<td>LON2-TspRI</td>
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<td>TspRI</td>
<td>159, 131, 28</td>
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<td>lon2-9</td>
<td>LON2-HpaI</td>
<td>TCAACCTATCCACCTCTTCTTAGACC</td>
<td>HpaII</td>
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<td>LON2-24</td>
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<td>lon2-10</td>
<td>R973-1</td>
<td>TCAGCGTCACACTAGTGGAAGCAAGGACGAT</td>
<td>Ncol</td>
<td>88, 27, 115</td>
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<td>R973-2</td>
<td>GGAATTAATCAGTGCCCAATTTGAAGAACCC</td>
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<td>lon2-277</td>
<td>LON2-22</td>
<td>CAGCGGTATGACGTATAGCAATGAAC</td>
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<td>221, 465, 686</td>
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<td>LON2-23</td>
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<td>lon2-310</td>
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<td>Mnll</td>
<td>100, 68, 32</td>
</tr>
<tr>
<td></td>
<td>LON2-Mnll</td>
<td>TGAAGATGCAGACAGCAGCTGCTACCC</td>
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<td></td>
</tr>
</tbody>
</table>

1 Marker from Burkhart et al., 2013
2 Marker from Rinaldi et al., 2016
2.1.4. Mutant isolation

1.3 g of seeds (~65,000 seeds) of lon2-2 35S:GFP-PTS1 were mutagenized for 16 hours at room temperature with 0.24% to 0.25% (v/v) ethylmethane sulfonate, rinsed extensively, and grown in 104 M₁ pools. For screening, 0.1 g of M₂ seeds (~5000 seeds) per pool were surface-sterilized, stratified, and plated on PNS medium supplemented with 8 μM IBA and solidified with 1.0% (w/v) agar in square Petri dishes (89 x 89 mm). Plates were positioned vertically, and seedlings were grown under yellow-filtered light for 8 days, when M₂ seedlings with three or more lateral roots were moved to soil. Leaf tissue was collected from approximately 30-day-old M₂ plants for immunoblot analysis using α-PMDH, α-NBR1, α-ATG7, and α-HSC70 antibodies (as described below in 2.5). M₃ seeds from M₂ plants with multiple lateral roots, complete PTS2 processing, and elevated NBR1 protein levels were collected and retested for IBA resistance (as described above in 2.1.2), and 6-day-old tissue was tested by immunoblot analysis using α-thiolase, α-ATG3, α-HSC70, and α-ICL or α-MLS (as described below in 2.5). Lines exhibiting IBA sensitivity and thiolase and ICL or MLS stabilization were retained as lon2 suppressors. All suppressors were genotyped to confirm lon2-2 homozygosity (Table 2.1). After suppressor mutation identification, PCR-based genotyping markers designed using dCAPS Finder 2.0 (Neff et al., 2002) were used to confirm the presence of the identified lesions (Table 2.3) and follow the mutations in the progeny of crosses.

2.2 Whole-genome sequencing

Genomic DNA from 13 lon2-2 suppressors was prepared for whole-genome sequencing. Genomic DNA from seven suppressors (atg2-6, atg5-6, atg5-7, atg7-19, atg16-1, atg16-2, and atg16-3) was prepared from F₂ seedlings from lines backcrossed to lon2-2 35S:GFP-PTS1. Surface-sterilized F₂ seeds were plated on PNS medium supplemented with 8 μM IBA and solidified with 0.6% (w/v) agar and grown at 22 °C under light filtered with yellow long-pass filers. At 10 d, between 50 and 100 F₂ seedlings with lateral roots were moved to sterile filter paper atop PNS medium and grown at 22 °C under white light for approximately 15 more days before collecting tissue. Genomic DNA from one suppressor (atg5-5) was prepared from F₃ seedlings pooled from three F₃ lines from a backcross to lon2-2 35S:GFP-PTS1, and genomic
<table>
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<tr>
<th>Mutant</th>
<th>Primer name</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Restriction Enzyme</th>
<th>Fragment size (bp)</th>
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<td>EcoRI</td>
<td>wt: 120, 199</td>
</tr>
<tr>
<td>atg7-15</td>
<td>ATG7-31</td>
<td>ACCGGTGTCACTTCTACTGTAATCTCTG</td>
<td>EcoRI</td>
<td>wt: 120, 199</td>
</tr>
<tr>
<td></td>
<td>ATG7-DpnII-L66</td>
<td>AACAATCTCGTGTTATGGTGTTGTTG</td>
<td>EcoRI</td>
<td>wt: 120, 199</td>
</tr>
<tr>
<td>atg7-16</td>
<td>ATG7-32</td>
<td>AGTCTGCTGATTTGTGTTTGTGTAATCTGA</td>
<td>EcoRI</td>
<td>wt: 120, 199</td>
</tr>
<tr>
<td>atg7-17</td>
<td>ATG7-25</td>
<td>CATGCAAACAGAAATACAGAGAGACTCG</td>
<td>EcoRI</td>
<td>wt: 120, 199</td>
</tr>
<tr>
<td>atg7-18</td>
<td>ATG7-Rev4</td>
<td>CAAGAAAAAGAGTGTTGCTGACGAGAAGAGAATCAGGAGACT</td>
<td>EcoRI</td>
<td>wt: 120, 199</td>
</tr>
<tr>
<td>atg7-19</td>
<td>ATG7-For5</td>
<td>TGGCTATGCTGACTCCCTACTCTTCTTGAG</td>
<td>EcoRI</td>
<td>wt: 120, 199</td>
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<tr>
<td>atg7-20</td>
<td>ATG7-6</td>
<td>AATCTCCTCTCAGTCAGTCTCAGTCTCAGT</td>
<td>EcoRI</td>
<td>wt: 120, 199</td>
</tr>
<tr>
<td>atg7-21</td>
<td>ATG7-L52For</td>
<td>ATGGCAAAAGGCGACAAATCTGAGAGAGACTCG</td>
<td>EcoRI</td>
<td>wt: 120, 199</td>
</tr>
<tr>
<td></td>
<td>ATG7-L52Rev</td>
<td>CAAGCAGGCTGAGTCTCTTGAGAAGAGAATCAGGAGACT</td>
<td>EcoRI</td>
<td>wt: 120, 199</td>
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<tr>
<td>atg7-22</td>
<td>ATG7-92DdeI</td>
<td>CTCAGTTTCTCAGAGAATCAGCAGTCTCGTTGCC</td>
<td>EcoRI</td>
<td>wt: 120, 199</td>
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<tr>
<td>atg7-23</td>
<td>ATG7-6</td>
<td>GATTTCTGCAAGAGCTCTTCCTTCTAAG</td>
<td>EcoRI</td>
<td>wt: 120, 199</td>
</tr>
<tr>
<td>atg7-24</td>
<td>ATG7-L92BclI</td>
<td>CTCAGTTTCTCAGAGAATCAGCAGTCTCGTTGCC</td>
<td>EcoRI</td>
<td>wt: 120, 199</td>
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<tr>
<td>atg7-25</td>
<td>ATG7-6</td>
<td>GATTTCTGCAAGAGCTCTTCCTTCTAAG</td>
<td>EcoRI</td>
<td>wt: 120, 199</td>
</tr>
<tr>
<td>Mutant</td>
<td>Primer name</td>
<td>Primer sequence (5’ to 3’)</td>
<td>Restriction Enzyme</td>
<td>Fragment size (bp)</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>----------------------------</td>
<td>--------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>atg16-1</td>
<td>ATG16-L55</td>
<td>TTGAGTATCTGTATGCAGGTTCCAGATT</td>
<td>MseI</td>
<td>162, 29</td>
</tr>
<tr>
<td></td>
<td>ATG16-11</td>
<td>CAACTCCTTCTCTTGAAAATAGCC</td>
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</tr>
<tr>
<td></td>
<td>ATG16-3</td>
<td>CAGTGCAATTTGTAGTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>atg16-2</td>
<td>ATG16-L68A</td>
<td>TTTTCTGTTTTCCCTCTCATTCTGAAGC</td>
<td>HindIII</td>
<td>242, 212, 30</td>
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<td></td>
<td>ATG16-12</td>
<td>TCAGAAATGGAAGGAAAGAAACAGAAA</td>
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<td>107, 29</td>
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<tr>
<td></td>
<td>ATG16-L68B2</td>
<td>CCTCTCTTGTTAAATAGCCTTTGAAGAT</td>
<td>BglII</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>ATG16-13</td>
<td>TCTTTTCTCTCTCTCTTGTAATCGTG</td>
<td>Spel</td>
<td>163, 138, 25</td>
</tr>
<tr>
<td></td>
<td>ATG16-L53Spel</td>
<td>AAGAGAAGGAGTTGTTAATATGACTAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATG16-13</td>
<td>TCTTTTCTCTAATCTCTTGTAATCGTG</td>
<td>PstI</td>
<td>132, 31, 163</td>
</tr>
<tr>
<td></td>
<td>ATG16-L53Pstl</td>
<td>AAGAGAAGGAGTTGTTAATATGACCTG</td>
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<td></td>
</tr>
<tr>
<td>atg16-3</td>
<td>ATG18A-1</td>
<td>GCGGTGTTGTGCACTGGTGAGAT</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>ATG18A-Ddel</td>
<td>GAAAGAGAGTTCTCCGATACATCGGCCTT</td>
<td>Ddel</td>
<td>143, 113, 30</td>
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<td>atg18a-3</td>
<td>ATG18A-L154</td>
<td>CTTCCAACCACTGAACGAGCCACCTGA</td>
<td>BglII</td>
<td>123, 98, 31, 27</td>
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<tr>
<td></td>
<td>ATG18A-4</td>
<td>TTACCTTCAACCTTAGACGCATA</td>
<td></td>
<td>27</td>
</tr>
<tr>
<td>atg18f</td>
<td>ATG18F-3</td>
<td>CCGTCCTCTTTTGCGCTGTG</td>
<td>Alul</td>
<td>120, 149, 77</td>
</tr>
<tr>
<td></td>
<td>ATG18F-Alul</td>
<td>GACACAATATGACATACGAACCTGACGA</td>
<td></td>
<td>77, 29, 77</td>
</tr>
</tbody>
</table>
DNA from five suppressors (\textit{atg2-7, atg2-8, atg18a-3, and atg18a-4}) was prepared from unbackcrossed lines.

Genomic DNA for whole-genome sequencing was prepared as previously described (Thole et al., 2014). DNA was sequenced with Illumina HiSeqation 2000 sequencers at the Genome Technology Access Center at Washington University in St. Louis and aligned with the TAIR 10 build of the \textit{A. thaliana} Col-0 genome using Novoalign (Novocraft; http://novocraft.com). SNPs were identified using SAMtools (Li et al., 2009) and annotated with snpEFF (Cingolani et al., 2012). Mutations were then filtered using a script that prioritized homozygous canonical EMS-derived mutations (G-to-A and C-to-T) resulting in non-synonymous amino acid changes or altered splice sites but also retained mutations in introns and heterozygous EMS-consistent mutations. We disregarded mutations that were present in our lab stock of Col-0 or in multiple suppressors from different pools, indicating an origin in the starting line.

Sequencing genomic DNA from the L1 isolate revealed mutations in both \textit{ATG18a} (c463t causing Q155Stop) and \textit{ATG18f} (g708a causing A198T). To determine which of these lesions was linked to \textit{lon2} suppression, I crossed L1 to \textit{lon2-2 35S:GFP-PTS1} and used genotyping markers (Table 2.3) to isolate F\textsubscript{3} plants that were homozygous for the \textit{ATG18a} lesion but homozygous for wild-type \textit{ATG18f} and vice versa. The resultant F\textsubscript{4} progeny were tested for IBA responsive lateral rooting and 6-day-old immunoblot phenotypes to reveal that the \textit{ATG18a} mutation suppressed \textit{lon2-2} whereas the \textit{ATG18f} mutation did not alter \textit{lon2-2} phenotypes. I used the backcrossed \textit{lon2-2 atg18a-3} mutant in subsequent analyses.

2.3. Individual gene sequencing

\textit{ATG3} or \textit{ATG7} was sequenced directly in suppressors with reduced ATG3 or ATG7 protein levels, respectively. \textit{ATG3} or \textit{ATG7} was PCR amplified using the primers indicated in Table 2.4. Amplicons were purified using Zymo PCR purification kit (Zymo Research) and sequenced directly (Lone Star Labs, Houston, TX or Genewiz, Houston, TX) with the primers used for amplification.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer name</th>
<th>Primer sequence (5' to 3')</th>
<th>Amplicon size (bp)</th>
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<tbody>
<tr>
<td>ATG3</td>
<td>ATG3-5</td>
<td>GTATTACCAAACCTCGTG</td>
<td>914</td>
</tr>
<tr>
<td></td>
<td>ATG3-6</td>
<td>ATGAAGATAATAGACGTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATG3-7</td>
<td>CGATACGATAAATTCTTCCTC</td>
<td>1020</td>
</tr>
<tr>
<td></td>
<td>ATG3-8</td>
<td>CAAGTTGAATAAGGATAAGTGAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATG3-9</td>
<td>CTGGCTACACATGGGAAC</td>
<td>932</td>
</tr>
<tr>
<td></td>
<td>ATG3-10</td>
<td>GACGATCACAATAATACCTC</td>
<td></td>
</tr>
<tr>
<td>ATG7</td>
<td>ATG7-27</td>
<td>CGTCATCGTCTTTCTTTCTCACATTCC</td>
<td>914</td>
</tr>
<tr>
<td></td>
<td>ATG7-28</td>
<td>GAACGAAACCTCAGGAACCTC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATG7-29</td>
<td>CTTAGCTCGGTGACTTGTGTTTTACTG</td>
<td>985</td>
</tr>
<tr>
<td></td>
<td>ATG7-30</td>
<td>TAAATCGACAGCAAAACAGCAAGCAACCT</td>
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<td></td>
<td>ATG7-31</td>
<td>ACCCGTGTCACCTCTAGTAACTCTG</td>
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<tr>
<td></td>
<td>ATG7-32</td>
<td>AGTCTCTGTGTATTGTTGTTTTATGTCA</td>
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<td></td>
<td>ATG7-33</td>
<td>CCGATAAAAGAAAGCAGTGGTT</td>
<td>1039</td>
</tr>
<tr>
<td></td>
<td>ATG7-34</td>
<td>GTCTATGGACATTTGATAAATCTGTC</td>
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</tr>
</tbody>
</table>
2.4. Plasmid subcloning and plant transformation

2.4.1. LON2 site-directed mutagenesis and subcloning

To generate lon2 variant lines, a LON2 cDNA in pBluescriptSK (stock pdx26935) was ordered from RIKEN. LON2 was subcloned from pBluescriptSK into another vector by Lisa Farmer, and I used DNA from this subclone to amplify LON2 cDNA using Q5 High-Fidelity DNA polymerase (New England BioLabs) with the LON2-Forward (or LON2-274-F for generating truncated lon2) and LON2-Reverse primers (Table 2.5). The LON2 cDNA fragment was gel purified and cloned into pENTR/D-TOPO (Invitrogen) following the instructions provided in the cloning kit. Site-directed mutagenesis was performed on the resultant vector using Q5 High-Fidelity DNA polymerase (New England BioLabs) with the primers listed in Table 2.5 to generate the indicated mutation and including restriction sites that were added to verify the presence of the desired mutation. The resultant pENTR-LON2 variants were cloned using LR Clonase II (Invitrogen) into pEarleyGate destination vectors pB7WG2F (Karimi et al., 2002) and pEG201 (Earley et al., 2006) from the ABRC to generate 35S:GFP-LON2 and 35S:HA-LON2 vectors. LON2 cDNA in each vector was sequenced using the primers listed in Table 2.6 to check verify the presence of the desired mutations and to verify insertion direction.

2.4.2. NBR1 subcloning

To generate NBR1 overexpressing lines, an NBR1 cDNA in pENTR223 (stock G25119) from the ABRC was cloned using LR Clonase II (Invitrogen) into pEarleyGate destination vectors pEG100, pEG104, and pEG201 (Earley et al., 2006) from the ABRC to generate 35S:NBR1, 35S:YFP-NBR1, and 35S:HA-NBR1 vectors. NBR1 cDNA in each vector was sequenced using the primers listed in Table 2.6 to check for possible mutations and to verify insertion direction.

2.4.3. Ubiuitin-GFP-mCherry-PEX26TM subcloning

To generate peroxisome membrane reporters, we ordered a synthesized, codon-optimized construct from GenScript that encodes the tandem-fluorescent peroxisome marker comprised of a non-cleavable ubiquitin, GFP, mCherry, and the transmembrane domain of PEX26 (PEX26TM) (UGMT) in pUC57. The UGMT construct was amplified using Q5 High-Fidelity
Table 2.5 Primers for *LON2* site-directed mutagenesis

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primer Name</th>
<th>Sequence (modified bases are in red and underlined)</th>
<th>Restriction site added</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>LON2-Forward</td>
<td>CACCATGGCGGAAACAGTGGAGCTCCCGAG</td>
<td>None</td>
</tr>
<tr>
<td>274-888</td>
<td>LON2-274-F</td>
<td>CACCATGAAATCAGAGGTAGTTCCCGCCATGG</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>LON2-Reverse</td>
<td>TCATAATTTAGAGTTTTGCTCCCGCGCCATGG</td>
<td></td>
</tr>
<tr>
<td>Y450A</td>
<td>LON2-Y450A-F1</td>
<td>GAGGACACAGAGAGAACAGCTATAGGAAAGCATGCCAGGCAGG</td>
<td>Alu</td>
</tr>
<tr>
<td></td>
<td>LON2-Y450A-R2</td>
<td>CCTATAGCTGTCTTCTGTGTCTCATTGTCAGC</td>
<td></td>
</tr>
<tr>
<td>E476Q</td>
<td>LON2-E476Q-F3</td>
<td>GGATCGATCTGATAAAACAGGCACGTTCCGAGG</td>
<td>BspDI/Clal</td>
</tr>
<tr>
<td></td>
<td>LON2-E476Q-R1</td>
<td>GTTTATACTGATCTGATCCACACAGCATAACTGGATGCAACACACC</td>
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<tr>
<td>S783A</td>
<td>LON2-S783A-F31</td>
<td>CCCGCTGCAGGCGGAGTGACTTTGGTGACAGGTTTGGTTCCGAGG</td>
<td>PstI</td>
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<td></td>
<td>LON2-S783A-R31</td>
<td>CCCGCTGCAGGCGGAGTGACTTTGGTGACAGGTTTGGTTCCGAGG</td>
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</tr>
</tbody>
</table>
Table 2.6 Primers for sequencing *LON2* and *NBR1*-containing plasmids

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer name</th>
<th>Primer sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LON2</strong></td>
<td>LON2-10s</td>
<td>GAAACAGTTGAGCTCCCGAGTAGTTAGCG</td>
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<tr>
<td></td>
<td>R973-2</td>
<td>GGATTAATCATCGTCCCAATTGAAGAACC</td>
</tr>
<tr>
<td></td>
<td>R082</td>
<td>AAAGATGCACCTCAGCAACCTGG</td>
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<tr>
<td></td>
<td>LON2-7s</td>
<td>GGCTAAACCATACTAGTCACCTGCAAGACG</td>
</tr>
<tr>
<td></td>
<td>LON2-5s</td>
<td>CAGATGGCGCATAGCTAATCTAAG</td>
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<td>LON2-2s</td>
<td>CCACTCCACTTTTCTCTGCTGG</td>
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<td></td>
<td>LON2-3s</td>
<td>CTTATCTTTGATGCCACCAACAGGCAGAAC</td>
</tr>
<tr>
<td><strong>NBR1</strong></td>
<td>NBR1-5</td>
<td>GGCAACCACATCCCCATCCTCATCAG</td>
</tr>
<tr>
<td></td>
<td>NBR1-6</td>
<td>AGGTCTTGTCTGGTTGTTCTCATACTAC</td>
</tr>
<tr>
<td></td>
<td>NBR1-7</td>
<td>CTTCCTTGTTCCTCAGCAGTCTC</td>
</tr>
<tr>
<td></td>
<td>NBR1-8</td>
<td>TATGGAGGTGCTTTAGGCTTTCA</td>
</tr>
<tr>
<td></td>
<td>NBR1-9</td>
<td>TAGTCCTGTAGTGAGAGTGCTTGGTGG</td>
</tr>
<tr>
<td></td>
<td>NBR1-10</td>
<td>GAGAATGACCGTGAGGATAGGAGATGAG</td>
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</table>
DNA polymerase (New England BioLabs) with the UGMT-Forward and UGMT-Reverse primers (Table 2.7). The UGMT fragment was gel purified and cloned into pENTR/D-TOPO (Invitrogen) following the instructions provided in the cloning kit. The ubiquitin and GFP components were removed using XbaI and AvrII, respectively, to generate pENTR-GFP-mCherry-PEX26TM (pENTR-GMT) and pENTR-mCherry-PEX26TM (pENTR-MT), respectively. The resultant pENTR-UGMT, pENTR-GMT, and pENTR-MT were cloned using LR Clonase II (Invitrogen) into pEarleyGate destination vectors pEG100 (Earley et al., 2006) and pUBQ10:GW (Michniewicz et al., 2015) to generate 35S:UGMT, 35S:GMT, 35S:MT, pUBQ10:UGMT, pUBQ10:GMT, and pUBQ10:MT plasmids. Each plasmid was sequenced using the primers listed in Table 2.7 to verify insertion direction and to ensure no undesired mutations were present.

2.4.4. Agrobacterium tumefaciens transformation and plant transformation

The resultant LON2, NBR1, and UGMT plamids described above were used to transform Agrobacterium tumefaciens GV3101 (pMP90) (Koncz and Schell, 1986) by electroporation. Transformed A. tumefaciens strains were used to transform Col-0 plants using the floral dip method (Clough and Bent, 1998). Transformed plants were selected for glufosinate ammonium (Basta) resistance. Overexpression of NBR1 was confirmed by immunoblot analysis using α-NBR1 (Svenning et al., 2011), and expression UGMT, GMT, or MT was confirmed by immunoblot analysis using α-mCherry (Novus NBP1-96752). The presence of the UGMT, GMT, or MT was verified using the primers in Table 2.7 as PCR-based genotyping markers. Homozygous progeny were used for phenotypic analysis.

2.5. Immunoblot analysis

Extracts were prepared from pooled seedlings of the indicated ages or adult leaves by homogenizing frozen tissue in 2 volumes of 2X sample buffer (Invitrogen) containing 0.05 M DTT and heating at 100 °C for 5 min. Samples were electrophoresed on Bolt 10% Bis-Tris Plus gels (Invitrogen) or NuPAGE 10% Bis-Tris Midi gels (Invitrogen) using 1X MOPS running buffer (50 mM MOPS, 50 mM Tris base, 0.1% SDS, and 1 mM EDTA) and transferred to Hybond Nitrocellulose membrane (Amersham Pharmacia Biotech) for 40 min using NuPAGE transfer buffer (Invitrogen). Membranes were blocked in 8% non-fat dry milk solution (or 5%
BSA for the α-ATG3 and α-ATG5 antibodies) in 20 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween 20 and then incubated overnight at 4 °C with primary antibodies in blocking solution. Rabbit antibodies against ATG3 (1:10,000; Phillips et al., 2008), ATG5 (1:2000, Agrisera AS15 3060), ATG7 (1:1000; Doelling et al., 2002), ICL (1:1000; Maeshima et al., 1988), mCherry (1:2000; Novus NBP1-96752), mitochondrial ATP synthase α–subunit (1:2000; MitoScience MS507) MLS (1:25,000; Olsen et al., 1993), NBR1 (1:2000; Svenning et al., 2011), the PED1 isoform of thiolase (1:20,000; Lingard et al., 2009), and PMDH2 (1:5000; Pracharoenwattana et al., 2007) were diluted as indicated. Mouse antibodies against HSC70 (1:100,000; StressGen Bioreagents SPA-817) were used. Primary antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies diluted in blocking buffer as follows: goat anti-rabbit (1:5000; Santa Cruz Biotechnology SC-2030 or GenScript A00098) or goat anti-mouse (1:5000; Santa Cruz Biotechnology SC-516102). Horseradish peroxidase was visualized with ProSignal Pico reagent (Prometheus Protein Biology Products) and autoradiography film. Membranes were reblocked and sequentially probed with the indicated antibodies without stripping the membrane between incubations.

2.6. Confocal fluorescence microscopy

2.6.1 Confocal microscopy of atg18a-3 GFP-PTS1

lon2-2 atg18a-3 35S:GFP-PTS1 was backcrossed to lon2-2 35S:GFP-PTS1 to reduce the number of background mutations and then crossed to 35S:GFP-PTS1 to remove the lon2-2 T-DNA insertion. Wild-type 35S:GFP-PTS1 and atg18a-3 35S:GFP-PTS1 seedlings grown on PNS under continuous white light were mounted in water, and fluorescence was visualized using a Carl Zeiss LSM 710 laser scanning confocal microscope equipped with a meta detector. Samples were imaged using a 63X oil immersion objective. GFP and chlorophyll were excited with a 488-nm argon laser. GFP emission was collected between 493 nm and 572 nm, and chlorophyll autofluorescence was collected between 620 nm and 719 nm. Each image is an average of two or four exposures using a 47-µm pinhole, corresponding to a 0.8-µm optical section.
### Table 2.7 Primers for sequencing and genotyping ubiquitin-GFP-mCherry-PEX26TM constructs

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>UBQ10-3</td>
<td>CCGTGATCAAGGTAATTTCTGTGTTCC</td>
</tr>
<tr>
<td>3S-F</td>
<td>GGATGACGCACAATCCCAATCCTCG</td>
</tr>
<tr>
<td>UGMT-Forward</td>
<td>CACCTCTAGAATGCAGATATCCGTGAAACCTTG</td>
</tr>
<tr>
<td>UGMT-1</td>
<td>CAGTAAACAATCTCTACCTTTAATACCA</td>
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<tr>
<td>UGMT-2</td>
<td>ACGTTTACATTATGGCTGATAAGGAAAAAAGA</td>
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<td>UGMT-3</td>
<td>CACAGATCCCTCCATATGAAACCTTGACCTC</td>
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<td>UGMT-4</td>
<td>TTAAGACAAACCTAAAAGCAAGGAAACCAGT</td>
</tr>
<tr>
<td>UGMT-Reverse</td>
<td>CCGGATTATGTAGTTGTAGATGAGGATTTGA</td>
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</tbody>
</table>

![Diagram showing the constructs and primers](image-url)
2.6.2. Confocal microscopy of mCherry-PEX26TM and lon2-8 GFP-PTS1 mCherry-PEX26TM

Wild-type pUBQ10: mCherry-PEX26TM and lon2-8 35S: GFP-PTS1 pUBQ10: mCherry-PEX26TM seedlings grown on PNS under continuous white light were mounted in water, and fluorescence was visualized using a Carl Zeiss LSM 710 laser scanning confocal microscope equipped with a meta detector. Samples were imaged using a 63X oil immersion objective. GFP was excited with a 488-nm argon laser. GFP emission was collected between 494 nm and 532 nm. mCherry was excited with a 543-nm argon laser. mCherry emission was collected between 580 nm and 629 nm. Each image is an average of two exposures using a 95.1-µm pinhole, corresponding to a 1.8-µm optical section.

2.6.3. Confocal microscopy of GFP-ATG8a

Wild-type 35S: GFP-ATG8a (Phillips et al., 2008) and lon2-2 35S: GFP-ATG8a seedlings grown on PNS under continuous white light were mounted in water, and fluorescence was visualized using a Carl Zeiss LSM 710 laser scanning confocal microscope equipped with a meta detector. Samples were imaged using a 63X oil immersion objective. GFP was excited with a 488-nm argon laser. GFP emission was collected between 494 nm and 532 nm. Each image is an average of two exposures using a 95.1-µm pinhole, corresponding to a 1.8-µm optical section.

2.6.4. Confocal microscopy of GFP-mCherry-PEX26TM

Wild-type pUBQ10: GFP-mCherry-PEX26TM seedlings grown on PNS under continuous white light were mounted in water, and fluorescence was visualized using a Carl Zeiss LSM 710 laser scanning confocal microscope equipped with a meta detector. Samples were imaged using a 63X oil immersion objective. GFP was excited with a 488-nm argon laser. GFP emission was collected between 494 nm and 532 nm. mCherry was excited with a 543-nm argon laser. mCherry emission was collected between 580 nm and 627 nm. Each image is an average of two exposures using a 95.1-µm pinhole, corresponding to a 1.8-µm optical section.

2.6.5. Confocal microscopy of ubiquitin-GFP-mCherry-PEX26TM

Wild-type pUBQ10: ubiquitin-GFP-mCherry-PEX26TM seedlings grown on PNS under continuous white light were mounted in water, and fluorescence was visualized using a Carl
Zeiss LSM 710 laser scanning confocal microscope equipped with a meta detector. Samples were imaged using a 63X oil immersion objective. GFP was excited with a 488-nm argon laser. GFP emission was collected between 495 nm and 539 nm. mCherry was excited with a 543-nm argon laser. mCherry emission was collected between 580 nm and 635 nm. Each image is an average of two exposures using a 47.1-µm pinhole, corresponding to a 0.9-µm optical section.

2.7. Statistical analysis

One-way ANOVA with Duncan’s test was used to assess statistical significance (SPSS Statistics software, Version 24.0.0.0).

2.8. Accession numbers

Sequence data can be found in the Arabidopsis Genome Initiative under the following accession numbers: At3g19190 (ATG2), At5g61500 (ATG3), At5g17290 (ATG5), At5g45900 (ATG7), At4g30790 (ATG11), At5g50230 (ATG16), At3g62770 (ATG18a), At5g47040 (LON2), and At4g24690 (NBRI). The Brassicaceae ATG2 homologs used for alignment in Fig. 3.5B have the following accession numbers: EFH59425.1 (Arabidopsis lyrata), XP_013585639.1 (Brassica oleracea), XP_009145821.1 (Brassica rapa), XP_006296813.1 (Capsella rubella), and XP_006406527.1 (Eutreum salsugineum). The ATG7 homologs used for alignment in Fig. 3.12 have the following accession numbers: OAO92345.1 (A. thaliana), NP_001336161.1 (H. sapiens), NP_611350.1 (D. melanogaster), and KZV10910.1 (S. cerevisiae). The ATG5 homologs used for alignment in Fig. 3.13 have the following accession numbers: OAO90612.1 (A. thaliana), AGC52703.1 (H. sapiens), AAF46252.2 (D. melanogaster), and KZV07366.1 (S. cerevisiae).
Chapter 3: A facile screen for autophagy-defective mutants in Arabidopsis
Parts of this chapter are submitted for publication (Young et al., 2018).

3.1. Introduction

Although homology-based analyses have identified conserved ATG genes in plants, only a few ATG genes have emerged from forward-genetic screens in A. thaliana. We recently recovered several *atg* mutants by screening for suppressors of *lon2* mutants (Table 3.1; Farmer et al., 2013). Because screening for *lon2* suppressors affords a homology-independent method to uncover Arabidopsis autophagy components, I expanded the *lon2* suppressor screen with the assistance of undergraduate students Michael Passalacqua and Kevin Chappell and graduate student Roxanna Llinas. This effort yielded 26 alleles of six *ATG* genes, including two *atg3*, two *atg18a*, three *atg2*, three *atg5*, three *atg16*, and 13 *atg7* mutants. We did not recover *atg11* mutants in our screen, but using reverse genetics, I demonstrated that loss of *ATG11* incompletely suppresses *lon2* phenotypes. I also report that the selective autophagy receptor NBR1 is neither necessary for pexophagy in the *lon2* mutant nor sufficient for pexophagy in Arabidopsis seedlings.

3.2. Screening strategy

With the assistance of Kevin Chappell and Roxanna Llinas, I used a four-step forward-genetic screen to recover Arabidopsis *atg* mutants (Fig. 3.1).

First, to identify potential *atg* mutants, we screened approximately 500,000 8-day-old *lon2*-2 M$_2$ seedlings from 104 mutagenized pools for the presence of lateral roots on medium containing 8 µM IBA (Fig. 3.1A). Plant peroxisomes convert the auxin precursor indole-3-butyric acid (IBA) to the active hormone indole-3-acetic acid (IAA) through β-oxidation (Zolman et al., 2000; Strader et al., 2010; Strader and Bartel, 2011), and IBA treatment promotes abundant lateral root production (Zolman et al., 2000) due to the IAA that is produced. Unlike wild type, *lon2* scarcely forms lateral roots in response to IBA (Lingard and Bartel, 2009) because *lon2* peroxisomes are excessively degraded by pexophagy (Farmer et al., 2013), reducing the number of peroxisomes and thereby presumably decreasing IBA-to-IAA conversion.
Second, to eliminate false positives and mutants with elevated auxin levels or responsiveness, we moved seedlings with lateral roots to soil and collected leaf tissue from approximately 30-day-old plants for immunoblot analysis. We used antibodies recognizing NBR1, peroxisomal malate dehydrogenase (PMDH), and ATG7 (Fig. 3.1B) to identify suppressors with impaired autophagy and improved peroxisome function. The selective autophagy receptor NBR1 is degraded at a basal rate via autophagy and accumulates when autophagy is impaired (Svenning et al., 2011). PMDH is synthesized as precursor containing an N-terminal peroxisomal-targeting signal 2 (PTS2) that is removed following peroxisomal import. *lon2-2* plants display PTS2 processing defects (Lingard and Bartel, 2009) presumably because elevated pexophagy reduces the number of peroxisomes, and preventing autophagy restores PMDH processing in *lon2-2* by increasing the number of import-competent peroxisomes (Farmer et al., 2013). M$_2$ plants with elevated NBR1 levels and restored PMDH processing were prioritized as likely *atg* mutants, and plants with decreased ATG7 levels were candidate *atg7* mutants.

Third, to confirm suppression of *lon2-2* phenotypes, we collected progeny of putative suppressors, retested IBA responsiveness, and examined thiolase, MLS or ICL, and ATG3 levels in 6-day-old seedlings (Fig. 3.1C). Thiolase is a peroxisomal enzyme that is destabilized in *lon2-2* seedlings (Lingard and Bartel, 2009) and stabilized in *lon2-2 atg* mutants (Farmer et al., 2013). Similarly, the peroxisomal enzymes ICL and MLS are stabilized in *lon2-2 atg* double mutants (Farmer et al., 2013). Mutants with elevated thiolase and ICL or MLS were prioritized as likely *atg* mutants, and plants with decreased ATG3 levels were candidate *atg3* mutants.

Fourth, to identify causal lesions, we either sequenced *ATG7* or *ATG3* in suppressors with reduced levels of the respective proteins or prepared DNA for whole-genome sequencing of the remaining suppressors (Fig. 3.1D). Michael Passalacqua assisted with characterization and sequencing of *ATG7* in candidate *atg7* mutants. Using this screen, we recovered 26 mutants carrying mutations in six different *ATG* genes (Table 3.1).
Figure 3.1 Four-step screening strategy for Arabidopsis autophagy-defective mutants. (A) Initial screening for lon2 suppressors. *lon2* forms few lateral roots in response to IBA; putative suppressors that formed lateral roots in the presence of IBA similar to wild-type and *lon2-atg7-4* were moved to soil for propagation. Top panel: wild-type (wt), *lon2-2*, and *lon2-2 atg7-4* seedlings were grown on media containing 8 µM IBA and imaged at 8 days; scale bar = 5
mm. Bottom row: magnified images of roots outlined in the top panel showing lateral roots, which are absent in lon2-2; scale bar = 1 mm.

(B) Secondary screening of putative suppressors. Leaf extracts from approximately 30-day-old adult controls (left of dashed line) or M₂ putative suppressor plants (right of dashed line) were processed for immunoblotting with antibodies to the indicated proteins. NBR1 is a selective autophagy receptor that accumulates in atg mutants (Svenning et al., 2011). PMDH is synthesized as a precursor (p) that is processed to a mature form (m) in the peroxisome. HSC70 is a loading control. The asterisk indicates a protein cross-reacting with the ATG7 antibody.

(C) Retesting progeny of putative suppressors that displayed restored PTS2 processing in the M₂ generation. Top: lateral root density of 8-day-old controls (left of dashed line) or M₃ or M₄ suppressor seedlings (right of dashed line) grown without or with IBA. Error bars show standard deviations (n = 8). Statistically significant (p<0.0001) differences determined by one-way ANOVA are depicted by different letters above the bars. Bottom: extracts from 6-day-old controls (left of dashed line) or M₃ or M₄ suppressor seedlings (right of dashed line) were processed for immunoblotting with antibodies to the indicated proteins. Malate synthase (MLS), thiolase, and isocitrate lyase (ICL) are peroxisomal proteins that are stabilized when both LON2 and autophagy are defective (Farmer et al., 2013). Membranes from duplicate gels were serially probed with the indicated antibodies to obtain the top three and bottom four panels. The asterisks indicate proteins cross-reacting with the ATG7 antibody or the ICL antibody.

(D) Identifying mutations via whole-genome sequencing. The L40 suppressor was backcrossed to the original lon2-2 line, IBA-sensitive F₂ seedlings were selected, and genomic DNA from pooled F₃ seedlings was sequenced. Homozygous single nucleotide polymorphisms consistent with EMS mutagenesis (G/C to A/T transitions) and resulting in nonsynonymous mutations in coding regions, altering splice sites, or occurring in introns or untranslated regions are indicated by locus identifiers to the right of the five Arabidopsis chromosomes, which were displayed using The Arabidopsis Information Resource Chromosome Map Tool.
Table 3.1 atg mutant alleles recovered as lon2-2 suppressors

<table>
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<th>Gene (accession number)</th>
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1Alleles in color were identified in the iteration of the lon2-2 suppressor screen described in this chapter. Alleles in black were identified in the pilot lon2-2 suppressor screen (Farmer et al., 2013).

2In all but one case, multiple isolates of allelic mutations were from the same M2 pool, indicating that the isolates were likely siblings. The exception was atg7-12, which was isolated from two independent pools.

3atg7-6 was independently isolated in a pilot lon2-2 suppressor screen (Farmer et al., 2013).
3.3. New atg7 alleles recovered as lon2 suppressors

ATG7, which encodes an E1-like enzyme involved in ATG8 lipidation (Doelling et al., 2002), was the gene that we recovered most commonly in our lon2 suppressor screen (Fig. 3.2; Table 3.1). Most of these suppressors were initially identified as atg7 mutants due to reduced or undetectable ATG7 levels (Fig. 3.2C). atg7-19 displayed near wild-type levels of ATG7 and was identified by whole-genome sequencing (Fig. 3.3A). We identified six nonsense mutations (atg7-6, atg7-13, atg7-14, atg7-15, atg7-20, and atg7-21), four splice-site mutations (atg7-10, atg7-12, atg7-16, and atg7-18), and three missense mutations (atg7-11, atg7-17, and atg7-19) (Fig. 3.2A). Although primarily novel atg7 alleles were recovered, a few duplicate alleles were isolated. The atg7-12 splice-site allele was found in two independent M2 pools, and the atg7-6 nonsense allele was previously recovered in our pilot lon2 suppressor screen (Farmer et al., 2013). Moreover, the nonsense allele atg7-13 harbors a mutation in a different nucleotide of the same codon as the previously published allele atg7-5 (Fig. 3.2A); although non-allelic, both of these mutations result in the same protein modification (W119Stop). I found that all of the atg7 mutants restored IBA responsiveness to lon2-2 (Fig. 3.2B) and displayed the characteristic immunoblot phenotypes of 6-day-old lon2-2 atg double mutants: accumulated NBR1, stabilized MLS and thiolase, and restored PTS2 processing (Fig. 3.2C). The early nonsense and splice-site mutations are expected to encode null alleles, and, indeed, I did not detect ATG7 protein in these mutants (Fig. 3.2C). The three missense alleles (atg7-11, atg7-17, and atg7-19) and atg7-21, which harbors a late nonsense mutation, accumulated detectable atg7 protein (Fig. 3.2C) and could therefore retain partial function, but these mutants nevertheless suppressed lon2-2 as well as the other nonsense and splice-site alleles (Fig. 3.2B, C).

3.4. New atg3, atg5, and atg16 alleles recovered as lon2 suppressors

In addition to ATG7, I isolated novel alleles of three other genes involved in ATG8 lipidation. I recovered two mutations in ATG3, which encodes an E2-like enzyme that conjugates ATG8 to phosphatidylethanolamine (Yamaguchi et al., 2012). atg3-2 and atg3-3 displayed decreased ATG3 protein levels (Fig. 3.4C and not shown) and were identified as a splice-site mutation and a nonsense mutation, respectively (Fig. 3.4A), by sequencing ATG3.
**Figure 3.2** Numerous novel *atg7* alleles recovered as *lon2* suppressors.

(A) Diagram of the *ATG7* gene. Boxes and lines represent protein coding regions and introns, respectively. The ThiF-like adenylation domain is indicated. The positions of new *atg7* mutations identified as *lon2* suppressors are in blue; previously described EMS-derived *lon2* suppressors (Farmer et al., 2013) are in gray, and T-DNA insertion alleles (Doelling et al., 2002; Hofius et al., 2009; Lai et al., 2011) are indicated by triangles. *atg7*-9 is an unpublished allele (alias 8-30) from the pilot *lon2*-2 suppressor screen (Farmer et al., 2013) that carries a g2959a mutation in the intron 10 splice acceptor site. The sequence of the *atg7*-13 nonsense allele compared to *atg7*-5 and wild-type *ATG7* is shown below the gene diagram. aa, amino acids.

(B) Lateral root density of 8-day-old wild type (wt), *lon2*-2, *atg7*-4, and *lon2*-2 *atg7* seedlings grown without or with IBA. Error bars show standard deviations (n = 8). Statistically significant (p<0.0001) differences determined by one-way ANOVA are depicted by different letters above the bars.

(C) Extracts from 6-day-old (left of dashed line) or 8-day-old seedlings (right of dashed line) were processed for immunoblotting with antibodies to the indicated proteins. The asterisk indicates a protein cross-reacting with the ATG7 antibody.
I recovered three mutations in ATG5, which encodes a protein that is conjugated by the ATG12 ubiquitin-like protein to act as an E3-like enzyme in ATG8 lipidation (Thompson et al., 2005; Romanov et al., 2012). I used whole-genome sequencing to determine that atg5-5 harbored a splice-site mutation (Fig. 3.1D, Fig. 3.4A), that atg5-6 harbored a missense mutation (E323K, Fig. 3.3B, Fig. 3.4A) and that atg5-7 harbored a splice-site mutation (Fig. 3.3C, Fig. 3.4A). atg5-5 and atg5-6 displayed reduced ATG5 protein levels (Fig. 3.4C).

I recovered three mutations in ATG16, which encodes a protein that tethers the ATG12–ATG5 conjugate to the membrane to allow ATG8 lipidation and autophagosome formation (Romanov et al., 2012). Whole-genome sequencing revealed that atg16-1 and atg16-3 harbored nonsense mutations (W47Stop and Q97Stop, respectively) and that atg16-2 harbored both a splice-site mutation and a missense mutation (R80K) in ATG16 (Fig. 3.3D-F, Fig. 3.4A); I surmise that the atg16-2 splice site mutation is causal because it is expected to prevent full-length ATG16 accumulation and is 5’ of the missense mutation.

All of the atg3, atg5, and atg16 alleles similarly restored lon2-2 IBA responsiveness (Fig. 3.4B) and displayed the characteristic immunoblot phenotypes of lon2-2 atg double mutant seedlings (Fig. 3.4C).

3.5. New atg2 alleles recovered as lon2 suppressors

I recovered mutants defective in ATG2, which is involved in expansion of the phagophore. I identified atg2-6 by sequencing genomic DNA from backcrossed seedlings, which revealed a cluster of mutations on chromosome 3 (Fig. 3.5A). Although ATG2 appeared in the middle of this region, the mutation in ATG2 was in intron 6 according to the primary prediction of Araport 11, the latest A. thaliana genome annotation. However, Araport 11 also predicts two additional ATG2 splice variants that would result in atg2-6 interrupting a splice-acceptor site. Indeed, the region of the intron following the mutated nucleotide can be translated in frame with exon 7 and lacks stop codons (Fig. 3.5B). Moreover, aligning predicted ATG2 proteins of other Brassicaceae family members revealed that this region of the predicted Arabidopsis intron 6 is conserved and exonic in other Brassicaceae ATG2 homologs (Fig. 3.5B), supporting the conclusion that the mutation in atg2-6 disrupts a splice site in A. thaliana and suggesting that the “alternative” splice variant encodes an ATG2 isoform that is essential for autophagy. Although the predicted splicing of A. lyrata ATG2 matched that of the A. thaliana ATG2 predicted by
Figure 3.3 Whole-genome sequencing of *lon2-2* suppressors reveals novel *atg7*, *atg5*, and *atg16* alleles.

The *lon2-2* suppressors (A) L71, (B) L44, (C) L56, (D) L65, (E) L68, and (F) L53 were backcrossed to the original *lon2-2* line, and genomic DNA from 50-100 pooled IBA-sensitive F2 seedlings was sequenced and analyzed as in the legend to Fig. 3.1D.
Figure 3.4 Novel atg3, atg5, and atg16 alleles recovered as lon2 suppressors.

(A) Diagrams of the ATG3, ATG5, and ATG16 genes. Boxes and lines represent protein coding regions and introns, respectively. The positions of new atg3, atg5, and atg16 mutations identified as lon2 suppressors are shown in pink, red, and orange, respectively; a previously described EMS-derived lon2 suppressor (Farmer et al., 2013) is in gray, and T-DNA insertion alleles (Thompson et al., 2005; Kulich et al., 2013; Ono et al., 2013) are indicated by triangles.

(B) Lateral root density of 8-day-old wild type (wt), lon2-2, atg3-1, lon2-2 atg3-2, lon2-2 atg5, and lon2-2 atg16 seedlings grown without or with IBA. Error bars show standard deviations (n = 8). Statistically significant (p<0.0001) differences determined by one-way ANOVA are depicted by different letters above the bars.

(C) Extracts from 6-day-old seedlings were processed for immunoblotting with antibodies to the indicated proteins. Membranes from duplicate gels were serially probed with the indicated antibodies to obtain the top five and bottom three panels. The asterisk indicates a protein cross-reacting with the ATG5 antibody.
Araport 11 (Fig. 3.5B), the A. *lyrata* genome was assembled based in part on alignment with A. *thaliana* (Hu et al., 2011), meaning that annotation errors in the A. *thaliana* genome may have been propagated to A. *lyrata*. Based on the whole-genome sequencing and Brassicaceae ATG2 alignment, I considered *atg2-6* as a splice-site mutation and the causal suppressor lesion.

I also used whole-genome sequencing to identify the *atg2-7* nonsense mutation (W90Stop, Fig. 3.5B, Fig. 3.6A) and the *atg2-8* nonsense mutation (W324Stop, Fig. 3.5B, Fig. 3.6B). *atg2-6* and *atg2-7* fully restored IBA responsiveness to *lon2-2* (Fig. 3.5C) and displayed the characteristic immunoblot phenotypes of *lon2-2* *atg* seedlings (Fig. 3.5D).

### 3.6. New *atg18a* alleles recovered as *lon2* suppressors

I recovered two mutants defective in ATG18a, which is also involved in expansion of the phagophore. Using whole-genome sequencing, I identified the *atg18a-3* nonsense mutation (Q155Stop, Fig. 3.5B, Fig. 3.7A) and the *atg18a-4* nonsense mutation (W364Stop, Fig. 3.5B, Fig. 3.7B). *atg18a-3* fully restored IBA responsiveness to *lon2-2* (Fig. 3.5C) and displayed the characteristic immunoblot phenotypes of *lon2-2* *atg* seedlings (Fig. 3.5D).

To further probe the importance of ATG18a in pexophagy, I backcrossed *lon2-2* *atg18a-3* to remove *lon2-2* and other unlinked mutations and examined *atg18a-3* seedlings constitutively expressing GFP carrying a peroxisomal targeting signal (GFP-PTS1; Zolman and Bartel, 2004) using confocal microscopy. *atg7* and *atg5* mutants accumulate peroxisomes in hypocotyls of developing seedlings (Kim et al., 2013), and *atg18a* mutants display clusters of oxidized peroxisomes in mesophyll cells (Shibata et al., 2013). I observed increased peroxisome abundance relative to wild type in *atg18a-3* cotyledon epidermal cells, cotyledon mesophyll cells, and hypocotyl cells in 4- to 7-day-old seedlings (Fig. 3.8), confirming that ATG18a promotes pexophagy during seedling development.

### 3.7. Loss of ATG11 partially suppresses *lon2* defects

Although ATG11 is important for autophagy in Arabidopsis (Li et al., 2014; Kang et al., 2018), no *atg11* mutants emerged from our *lon2-2* suppressor screen. To test the importance of ATG11 in *lon2*-related pexophagy, I crossed the *atg11-1* T-DNA insertional allele (Li et al., 2014) to *lon2-2*. Although the *lon2-2* PTS2-processing defect was fully suppressed in *lon2-2*
Figure 3.5 Novel \textit{atg2} and \textit{atg18a} alleles recovered as \textit{lon2} suppressors. 

(A) The L60 suppressor was backcrossed to the original \textit{lon2-2} line and genomic DNA from pooled IBA-sensitive F$_2$ seedlings was sequenced. Homozygous single nucleotide polymorphisms consistent with EMS mutagenesis (G/C to A/T transitions) resulting in nonsynonymous mutations in coding regions, altering splice sites, or occurring in introns or
untranslated regions are indicated by locus identifiers to the right of the five Arabidopsis chromosomes, which were displayed using The Arabidopsis Information Resource Chromosome Map Tool. (B) Diagrams of the ATG2 and ATG18a genes. Boxes and lines represent protein coding regions and introns, respectively. The positions of new atg2 and atg18a mutations identified as lon2 suppressors are shown in green and teal, respectively; positions of previously described EMS-derived lon2 suppressors (Farmer et al., 2013) are in gray, and T-DNA insertion alleles (Yoshimoto et al., 2009; Lenz et al., 2011) are indicated by triangles. The partial alignment shows predicted Brassicaceae ATG2 proteins including two A. thaliana ATG2 splice variants predicted by the latest genome annotation (Araport 11); the alternative ATG2 splice variant is interrupted by the atg2-6 mutation. aa, amino acids. (C) Lateral root density of 8-day-old wild type (wt), lon2-2, atg2-4, lon2-2 atg2, and lon2-2 atg18a-3 seedlings grown without or with IBA. Error bars show standard deviations (n = 8). Statistically significant (p<0.0001) differences determined by one-way ANOVA are depicted by different letters above the bars. (D) Extracts from 6-day-old seedlings were processed for immunoblotting with antibodies to the indicated proteins.
Figure 3.6 Whole-genome sequencing of lon2-2 suppressors reveals novel atg2 alleles. Genomic DNA from progeny of original (A) L79 and (B) L76 suppressor isolates was sequenced and analyzed as in the legend to Fig. 3.1D.
Figure 3.7 Whole-genome sequencing of _lon2-2_ suppressors reveal novel and _atg18a_ alleles. Genomic DNA from progeny of original (A) L1 and (B) L154 suppressor isolates was sequenced and analyzed as in the legend to Fig. 3.1D.
atg11-1 (Fig. 3.9B), atg11-1 only partially suppressed lon2-2 IBA resistance (Fig. 3.9A) and other immunoblot phenotypes (Fig. 3.9B). Thiolase was only partially stabilized in lon2-2 atg11-1, and MLS was not stabilized (Fig. 3.9B). This incomplete suppression suggests that some pexophagy still occurs in atg11-1. Additionally, NBR1 accumulated to intermediate levels in both atg11-1 and lon2-2 atg11-1 (Fig. 3.9B), which is consistent with only partially disrupted autophagy.

3.8. NBR1 is not necessary for pexophagy of lon2 peroxisomes

NBR1 is a selective autophagy receptor in Arabidopsis (Svenning et al., 2011; Zhou et al., 2013; Zhou et al., 2014) and is necessary and sufficient for pexophagy in mammals (Deosaran et al., 2013), but no nbr1 mutants emerged from our screen for lon2-2 suppressors. To test the necessity of NBR1 in pexophagy in Arabidopsis, I obtained T-DNA insertional alleles of nbr1 (Fig. 3.10A) and isolated lon2-2 nbr1-1 and lon2-2 nbr1-4 double mutants. Unlike the lon2-2 atg double mutants, these lon2-2 nbr1 double mutants resembled lon2-2 and did not form lateral roots in response to IBA (Fig. 3.10B). Moreover, nbr1 did not stabilize MLS or thiolase or suppress the PTS2-processing defects of lon2 (Fig. 3.10C). This lack of suppression suggested that NBR1 is dispensable for pexophagy in lon2.

If NBR1 were sufficient to induce pexophagy, then I expected that overexpressing NBR1 would stimulate pexophagy, as it does in mammalian cells (Deosaran et al., 2013), and phenocopy lon2. I drove untagged, HA-tagged, and YFP-tagged NBR1 from the constitutive cauliflower mosaic virus 35S promoter. Despite elevated NBR1 levels (Fig. 3.10D), these lines resembled wild type in lateral root formation (Fig. 3.10B), PTS2 processing (Fig. 3.10D), and thiolase stability (Fig. 3.10D), suggesting that NBR1 is not sufficient to induce pexophagy and supporting the conclusion that NBR1 is not a pexophagy receptor in Arabidopsis seedlings.

3.9. Causal lesion in lon2-2 suppressor L70 is unidentified

The causal lesion in lon2-2 suppressor L70 has not been identified. Similar to lon2-2 atg mutants, L70 forms lateral roots on IBA, accumulates NBR1, stabilizes ICL and thiolase, and does not have a PMDH PTS2-processing defect (not shown). Genomic DNA from progeny of original L70 suppressor isolates was sequenced and examined for single nucleotide
Figure 3.8 Increased peroxisome abundance in \textit{atg18a-3} seedlings. (A) Cotyledon epidermal cells, (B) cotyledon mesophyll cells, and (C) hypocotyl cells in 4-, 5-, 6-, and 7-day-old wild-type and twice backcrossed \textit{atg18a-3} seedlings expressing the peroxisomal matrix marker \textit{35S::GFP-PTS1} were imaged for GFP fluorescence (green) and chlorophyll autofluorescence (magenta) using confocal microscopy. Scale bars = 20 µm.
Figure 3.9 Loss of ATG11 partially suppresses lon2 defects.

(A) Lateral root density of 8-day-old wild type (wt), lon2-2, atg7-4, atg11-1, lon2-2 atg7-4, and lon2-2 atg11-1 seedlings grown without or with IBA. Error bars show standard deviations (n = 8). Statistically significant (p < 0.0001) differences determined by one-way ANOVA are depicted by different letters above the bars.

(B) Extracts from 4-, 6-, and 8-day-old seedlings were processed for immunoblotting with antibodies to the indicated proteins. An asterisk indicates a protein cross-reacting with the NBR1 antibody.
Figure 3.10 NBR1 is not necessary for pexophagy of lon2 peroxisomes and is not sufficient to induce pexophagy.

(A) Diagram of the NBR1 gene. Boxes and lines represent protein coding regions and introns, respectively. The PB1 domain, zinc finger, NBR1 domain, ATG8 binding site, and ubiquitin associating (UBA) sites are indicated. Triangles mark the locations of T-DNA insertion alleles.

(B) Lateral root density of 8-day-old wild type (wt), lon2-2, nbr1-1, atg7-3, lon2-2 nbr1, lon2-2 atg7-4, 35S:NBR1, 35S:HA-NBR1, and 35S:YFP-NBR1 seedlings grown without or with IBA. Error bars show standard deviations (n = 8). Statistically significant (p < 0.0001) differences determined by one-way ANOVA are depicted by different letters above the bars.

(C) and (D) Extracts prepared from 4- and 6-day-old seedlings were processed for immunoblotting with antibodies to the indicated proteins.
polymorphisms consistent with EMS mutagenesis, revealing 216 homozygous mutations resulting in nonsynonymous mutations in coding regions, altering splice sites, or occurring in introns or untranslated regions (Fig. 3.11). None of the 216 mutations disrupt any known ATG genes, hinting that the mutant may reveal a novel means to impede autophagy.

3.10. Discussion

Screening for lon2-2 suppressors revealed more than 20 novel alleles in six ATG genes (Table 3.1), providing new mutants for studying autophagy in Arabidopsis. A pilot lon2-2 suppressor screen uncovered atg2, atg3, and atg7 mutants (Farmer et al., 2013), and the expanded screen described here uncovered additional alleles of these genes plus atg5, atg16, and atg18a mutants. In contrast to our screen, a single atg2 allele emerged from a forward-genetic screen for plants with enhanced cell death in response to fungal infection (Wang et al., 2011), a single atg2 allele emerged from a hydroxyurea-resistance screen that primarily recovered catalase mutants (Hackenberg et al., 2013), and two atg2 alleles along with single atg7 and atg18a mutants were recovered from a microscopy-based screen for aggregated peroxisomes (Shibata et al., 2013). The lon2-2 suppressor screen combines high throughput with specificity and is the only forward-genetic screen to report atg3, atg5, or atg16 mutants. Although atg5 T-DNA insertional mutants have been characterized (Thompson et al., 2005; Kulich et al., 2013; Ono et al., 2013), no Arabidopsis atg3 or atg16 mutants have been reported aside from those arising from lon2-2 suppressor screens described here and previously (Farmer et al., 2013), and the alleles described here will be useful for future studies to determine if ATG3 and ATG16 function similarly in Arabidopsis as in other organisms.

Roughly half of the lon2-2 suppressors harbored a mutation in ATG7 (Table 3.1); the reason for this abundance of atg7 mutants is mysterious. When adding the atg alleles identified in our pilot screen (Farmer et al., 2013), a total of 18 of 34 lon2-2 suppressors were atg7 mutants, and atg7-6 was isolated in both iterations of the screen (Table 3.1) (Farmer et al., 2013). Although ATG7 encodes a relatively large protein (697 aa), ATG2 and ATG11 encode larger proteins (1861 and 1148 aas, respectively) and thus would seem to be more likely to be mutagenized, yet our combined screening recovered only five atg2 and no atg11 mutants. Moreover, ATG2 has 12 introns (Fig. 3.5B) compared to 10 introns in ATG7 (Fig. 3.2A), suggesting that ATG2 would present more opportunities than ATG7 for mutations in splice sites.
**Figure 3.11** Whole-genome sequencing of *lon2-2* suppressor L70.
Genomic DNA from progeny of original L70 suppressor isolates was sequenced and analyzed as in the legend to Fig. 3.1D.
in addition to coding regions. The frequency of atg7 mutants recovered in this screen might be explained by the structure and function of the ATG7 protein. As an E1-like enzyme, ATG7 activates both ATG8 and ATG12 and coordinates transfer to their respective E2-like enzymes, ATG3 and ATG10 (Yamazaki-Sato et al., 2003; Hong et al., 2011; Noda et al., 2011; Yamaguchi et al., 2012). ATG7 is the interaction hub among these proteins, requiring precise spatial arrangement (Hong et al., 2011; Noda et al., 2011; Yamaguchi et al., 2012), so it is tempting to speculate that ATG7 is more susceptible to functional perturbation via missense mutations than other ATG proteins. Indeed, five of the 18 atg7 mutants identified as lon2-2 suppressors (atg7-7, atg7-8, atg7-11, atg7-17, and atg7-19; Fig. 3.2A) harbor missense mutations whereas atg5-6 is our only non-atg7 lon2-2 suppressor to harbor a missense mutation. However, fewer mutations in other ATG genes were recovered, hindering comparison of the relative proportions of null versus missense alleles; further screening might recover missense alleles of other ATG genes. Moreover, even discounting missense mutations, 13 of our atg7 mutants harbor nonsense or splice-site mutations, which still more than doubles the number of alleles recovered for any other ATG gene (Table 3.1), indicating that the explanation for the apparent bias towards atg7 mutants may exist beyond the structure and function of ATG7. Perhaps the ATG7 locus is more accessible to EMS mutagenesis than other ATG genes.

Protein alignments of ATG7 and ATG5 provide some insight into how the recovered alleles might disrupt the function of the respective proteins. Four of the identified atg7 alleles have detectable atg7 protein: atg7-11, atg7-17, atg7-19, and atg7-21 (Fig. 3.2C). The latter of these alleles, atg7-21, harbors a late nonsense mutation (Q629Stop) whereas the other three alleles harbor missense mutations. Interestingly, atg7-7 and atg7-8 are missense alleles but do not express detectable atg7 protein (Farmer et al., 2013). An alignment of ATG7 proteins revealed that the mutations in the alleles with detectable atg7 protein altered residues that are conserved in plants, humans, fruit flies, and yeast (Fig. 3.12). In contrast, the mutations in atg7-7 and atg7-8 disrupt nonconserved residues, suggesting that these alleles were recovered because the mutations destabilize the proteins. The mutation in atg7-21 does not disrupt a conserved residue, but the nonsense mutation likely results in a truncated atg7 that is missing conserved Zn-coordinating cysteine residues, which may account for the apparent loss of function. Both atg7-11 and atg7-17 alter conserved glycine residues (Fig. 3.12) and express reduced atg7 protein levels (Fig. 3.2C), indicating that these mutations partially destabilize the proteins. The mutation
in \textit{atg7-19} alters a conserved threonine residue that neighbors the conserved active site cysteine residue (Fig. 3.12), and as \textit{atg7-19} expresses \textit{atg7} protein at a similar level to wild type (Fig. 3.2C), the altered threonine residue is likely important for proper formation of the active site. In addition to the \textit{atg7} missense alleles, \textit{atg5-6} is the only other missense allele that was recovered as a \textit{lon2-2} suppressor, and an alignment of ATG5 proteins revealed that the mutation in \textit{atg5-6} disrupts a conserved acidic residue (Fig. 3.13). Despite the conservation of the residue, I did not detect \textit{atg5-6} protein (Fig. 3.4C), indicating that the mutation destabilizes the protein, so no inferences can be drawn on the importance of the mutated residue for the function of ATG5.

ATG18a appears to act non-redundantly with the other seven ATG18 isoforms in Arabidopsis seedling pexophagy. The \textit{atg18a-3} nonsense allele fully restored \textit{lon2-2} IBA responsiveness and PTS2 processing, and \textit{lon2-2 atg18a-3} seedlings displayed similarly stabilized NBR1, MLS, and thiolase as other \textit{lon2-2 atg} double mutants (Fig. 3.5C, D). Moreover, a second \textit{atg18a} mutant was identified as a \textit{lon2-2} suppressor (Fig. 3.5B, 3.7). Similarly, an \textit{atg18a} mutant was recovered from a screen for aggregated peroxisomes (Shibata et al., 2013), and I observed increased peroxisome abundance in \textit{atg18a-3} (Fig. 3.8), which I attribute to decreased pexophagy. ATG18a functions beyond pexophagy; RNAi-based ATG18a downregulation confers hypersensitivity to starvation (Xiong et al., 2005) and various abiotic stresses (Xiong et al., 2007; Liu et al., 2009). Although most of the ATG18 isoforms are upregulated during seed maturation (Di Berardino et al., 2018), whether the remaining seven Arabidopsis ATG18 isoforms (Xiong et al., 2005) function in autophagy remains an open question.

Because no \textit{atg11} mutants emerged from our screen for \textit{lon2-2} suppressors, I crossed the \textit{atg11-1} T-DNA insertional allele to \textit{lon2-2} and observed only partial suppression of \textit{lon2-2} phenotypes (Fig. 3.9), suggesting that pexophagy is incompletely blocked in \textit{atg11-1}. Indeed, NBR1 is only partially stabilized in \textit{atg11-1} relative to \textit{atg7-4} (Fig. 3.9B), consistent with previous reports that autophagy is incompletely blocked in \textit{atg11-1} (Li et al., 2014; Kang et al., 2018). Moreover, \textit{atg11-1} accumulates less catalase than other \textit{atg} mutants (Kang et al., 2018), consistent with our conclusion that pexophagy is only partially disrupted when ATG11 is absent. As ATG11 is thought to act as a scaffold for ATG1a and ATG13 within the pre-autophagosomal structure (Li et al., 2014), these findings imply that autophagy can still occur, albeit less efficiently, in Arabidopsis when ATG11 is missing. Despite the large size of ATG11 (1148 aa),
Figure 3.12 ATG7 alignment highlighting structural features and atg7 missense alleles. For clarity, the diagram of the ATG7 gene in Fig. 3.2A is shown above the ATG7 alignment. ATG7 from Arabidopsis (A. thaliana; OAO92345.1), human (H. sapiens; NP_001336161.1), fruit fly (D. melanogaster; NP_611350.1), and budding yeast (S. cerevisiae; KZV10910.1) were aligned using the MegAlign program of DNASTar and the Clustal W method. Identical residues...
in three or more sequences are boxed in black; chemically similar residues are boxed in gray.
atg7 missense alleles isolated as lon2 suppressors are shown above the sequence in blue. Protein
features highlighted include the active site Cys residue (red) as well as several Zn-coordinating
Cys residues (dark orange) and nucleotide binding residues (light orange) revealed in the crystal
structure of the yeast ATG7 protein (Taherbhoy et al., 2011).
Figure 3.13 ATG5 alignment highlighting structural features and the atg5-6 missense allele. For clarity, the diagram of the ATG5 gene in Fig. 3.4A is shown above the ATG5 alignment. ATG5 from Arabidopsis (A. thaliana; OAO90612.1), human (H. sapiens; AGC52703.1), fruit fly (D. melanogaster; AAF46252.2), and budding yeast (S. cerevisiae; KZV07366.1) were aligned using the MegAlign program of DNAStar and the Clustal W method. Identical residues in three or more sequences are boxed in black; chemically similar residues are boxed in gray. The atg5-6 missense allele isolated as a lon2 suppressor is shown above the sequence in red. Protein features highlighted include the Lys residue (K128) in the helix-rich central domain (green) that is the conjugation site for ATG12 as well as the N-terminal and C-terminal ubiquitin-like (Ubl) domains revealed in the crystal structure of the yeast ATG5 protein (Matsushita et al., 2007).
its role as a scaffold may provide some tolerance for missense mutations, which, combined with the observation that \textit{atg11-1} only partially restored \textit{lon2-2} IBA responsiveness (Fig. 3.9A), may explain why \textit{atg11} mutants did not emerge from the screen for \textit{lon2-2} suppressors. In \textit{Pichia pastoris}, ATG11 binds to ATG8 and to the pexophagy receptor ATG30 (Farré et al., 2013), so it will be interesting to determine if ATG11 plays a similar role in Arabidopsis pexophagy. Arabidopsis ATG11 also interacts with ATG8 (Li et al., 2014), and additional studies are needed to decipher the role of this protein in processes upstream and downstream of ATG8 lipidation.

Besides \textit{ATG11}, mutants of five other single-copy Arabidopsis \textit{ATG} homologs did not emerge from our \textit{lon2-2} suppressor screen: \textit{ATG6, ATG9, ATG10, ATG101, and ATG20}. Of these six, only \textit{ATG20} has not been directly implicated in autophagy in plants. The predicted Arabidopsis \textit{ATG20} (At5g06140), also known as Sorting Nexin 1, acts in endosomal sorting of PIN2 (Jaillais et al., 2006), but a role for ATG20 in autophagy has not been established in plants. Conversely, although Arabidopsis \textit{ATG6} is important for autophagy (Patel and Dinesh-Kumar, 2008), \textit{atg6} defects in pollen germination result in male sterility (Fujiki et al., 2007; Qin et al., 2007) that would preclude recovery from our \textit{lon2-2} suppressor screen, which requires fertility. Arabidopsis \textit{ATG9} is a transmembrane protein critical for autophagosome formation (Hanaoka et al., 2002; Shin et al., 2014; Floyd et al., 2015; Zhuang et al., 2017), but autophagic flux is only partially compromised in \textit{atg9} mutants (Shin et al., 2014; Kang et al., 2018). It would be interesting to determine if \textit{atg9} mutants partially suppress \textit{lon2-2} similarly to \textit{atg11} mutants (Fig. 3.9). \textit{ATG10} is an E2-like enzyme that conjugates ATG12 during autophagosome formation and is essential for Arabidopsis autophagy (Phillips et al., 2008). \textit{ATG101} interacts with ATG11 and ATG13a (Li et al., 2014), but no \textit{atg101} mutants have been described. \textit{ATG10} (226 aa) and \textit{ATG101} (215 aa) are relatively small proteins, making smaller targets for EMS mutagenesis; further screening might yield mutants of these genes.

In addition to \textit{ATG18}, several \textit{ATG} genes are present as gene families in Arabidopsis: \textit{ATG1} (3 genes), \textit{ATG4} (2 genes), \textit{ATG8} (9 genes), \textit{ATG12} (2 genes), and \textit{ATG13} (2 genes). The roles of these genes in Arabidopsis autophagy have been experimentally validated using reverse-genetic and biochemical approaches (Doelling et al., 2002; Hanaoka et al., 2002; Chung et al., 2010; Suttangkakul et al., 2011; Woo et al., 2014). The failure of these genes to emerge from forward-genetic screens for \textit{lon2-2} suppressors is consistent with the possibility that, unlike \textit{ATG18a} (Fig. 3.5), these genes act redundantly in pexophagy.
Identification of the causal lesion in the \textit{lon2}-2 suppressor L70 may reveal a plant-specific gene important for autophagy or a gene specific to pexophagy. None of the 216 mutations identified by whole-genome sequencing of unbackcrossed L70 is in a known ATG gene. I have backcrossed L70, and whole-genome sequencing of pooled backcrossed L70 is expected to reduce the list of candidate causal lesions from 216 to a dozen or so. Whole-genome sequencing of other pooled backcrossed \textit{lon2}-2 suppressors revealed linkage to the causal lesion, which is typically in the middle of the cluster of mutated genes (Fig. 3.1D, Fig. 3.3, Fig. 3.5A). Efforts to identify the causal lesion will prioritize the mutations in the center of the cluster.

In spite of the conservation of autophagy across kingdoms, identified pexophagy receptors differ among species, and a pexophagy receptor has not been reported in plants. \textit{Pichia pastoris} Atg30 (Farré et al., 2008) and \textit{Saccharomyces cerevisiae} Atg36 (Motley et al., 2012) act as pexophagy receptors, but plants lack Atg30 or Atg36 homologs. We did not recover mutants of candidate pexophagy receptors in our screen for \textit{lon2}-2 suppressors. Because NBR1 is a pexophagy receptor in mammalian cells (Deosaran et al., 2013) and a selective autophagy receptor in Arabidopsis (Svenning et al., 2011; Zhou et al., 2013; Zhou et al., 2014; Hafrén et al., 2017), I used a reverse-genetics approach to investigate the role of Arabidopsis NBR1 in pexophagy. T-DNA insertional alleles of \textit{nbr1} failed to suppress \textit{lon2}-2 defects, and NBR1 overexpression failed to phenocopy \textit{lon2} seedling defects, which presumably result from heightened pexophagy (Fig. 3.10). These data suggest that NBR1 is not a pexophagy receptor in \textit{lon2} but do not eliminate the possibility that NBR1 acts redundantly in pexophagy or in other contexts. Intriguingly, peroxisomal catalase accumulates in \textit{nbr1} mutants during heat stress (Zhou et al., 2014), suggesting that NBR1 might be important for LON2-independent pexophagy in Arabidopsis. Further studies are needed to elucidate the roles of NBR1 in Arabidopsis selective autophagy.

Perhaps plants utilize a pexophagy receptor that is distinct from those identified in yeast and mammalian cells. For example, the plant-specific ATG8-INTERACTING (ATI) proteins (Honig et al., 2012; Zhou et al., 2018), are pexophagy receptor candidates, but mutations in these genes did not emerge from our screen for \textit{lon2}-2 suppressors. The ubiquitin-binding protein DSK2 (Lin et al., 2011) is another pexophagy receptor candidate in Arabidopsis. Arabidopsis DSK2 binds to and targets the transcription factor BES1 for degradation via autophagy (Nolan et al., 2017). DSK2 also interacts with two peroxisomal membrane proteins (Kaur et al., 2013).
Because Arabidopsis has two DSK2 paralogs (Farmer et al., 2010), recovering dsk2 mutants as lon2-2 suppressors is unlikely, and directed approaches will be needed to explore the role of ATI proteins and DSK2 in pexophagy.

Peroxin (PEX) proteins are necessary for peroxisome biogenesis and matrix protein import; many of these proteins are found on the peroxisome membrane where they would be accessible to the autophagy machinery. Several peroxins have been implicated in pexophagy in Arabidopsis as well as yeast and mammalian cells. Despite the potential importance of peroxins in pexophagy, pex mutants are unlikely to emerge as lon2-2 suppressors because pex mutants are generally IBA resistant (Kao et al., 2018) and thus not expected to restore IBA-responsive lateral rooting to lon2 (Fig. 3.1A). Alternative approaches will be needed to dissect the roles of peroxins in Arabidopsis pexophagy.

In summary, we used forward genetics to recover 26 additional alleles of six ATG genes. All six genes are core ATG genes, underscoring the functional conservation of these genes. These mutant alleles will be useful in future studies interrogating the role of these genes in Arabidopsis autophagy, especially the atg3 and atg16 alleles, which disrupt genes without available T-DNA insertional alleles. Using the lon2 mutant as a system for studying pexophagy in Arabidopsis, I also investigated the role of ATG11 and NBR1 in this process and found that ATG11 plays a limited role in pexophagy whereas NBR1 does not appear to be involved in lon2-related pexophagy. lon2 mutants will undoubtedly serve as a useful platform for future studies to elucidate the regulation of pexophagy in Arabidopsis.
Chapter 4: Characterization of the peroxisomal protease LON2 to provide insights into peroxisome quality control

4.1. Introduction

The peroxisomal protease LON2 plays a key role in peroxisome homeostasis. LON proteases are conserved AAA+ proteases involved in protein quality control and contain an N-terminal domain, an AAA domain, and a protease domain on a single polypeptide (reviewed in Iyer et al., 2004; Venkatesh et al., 2012). Recent research has uncovered interplay between LON2 and pexophagy in Arabidopsis and revealed that, like other LON proteases (reviewed in Gur, 2013; Voos et al., 2013), LON2 functions as both a chaperone and a protease. The discovery that atg mutants suppress lon2 defects (see Chapter 3; Farmer et al., 2013) suggests that pexophagy is elevated when LON2 is nonfunctional. Moreover, plants expressing a protease-inactive lon2 exhibit phenotypes similar to a lon2 atg double mutant (Goto-Yamada et al., 2014), indicating that the AAA domain of LON2 contributes to the prevention of pexophagy. Additionally, lon2 atg double mutants and plants expressing protease-inactive lon2 stabilize glyoxylate-cycle enzymes, indicating that LON2 also functions as a protease that degrades matrix proteins.

Many of the details regarding the mechanism of LON2 in enacting this quality control remain unclear. What are the substrates of LON2? Does LON2 act as a chaperone for some substrates and as a protease for other substrates? How does LON2 recognize its substrates? Mutating specific domains of E. coli Lon can yield Lon variants that remain capable of degrading a specific substrate but incapable of preventing proteotoxic stress and vice versa (Wohlever et al., 2014), indicating that these activities are separable. It is possible that LON2 may similarly utilize distinct mechanisms in peroxisome quality control. The large puncta present in lon2 mutants expressing peroxisome-localized GFP have not been explained but are suspected to be autophagy intermediates as the large-puncta phenotype is suppressed in lon2 atg double mutants (Farmer et al., 2013; Goto-Yamada et al., 2014). Although mutants defective in autophagy suppress lon2, no chemical autophagy inhibitors have been reported to suppress lon2, and lon2 mutants could serve as a platform to identify and assess chemical autophagy inhibitors, which would be useful for further studies on autophagy in Arabidopsis.
In this chapter, I describe my efforts to assess the functions of LON2 and enable further studies. First, I describe experiments in which I directly compare a series of lon2 alleles that have emerged from various forward-genetic screens in the Bartel lab and note different phenotypes amongst alleles, which is consistent with LON2 having distinct mechanisms in peroxisome quality control. Next, I provide confocal images of lon2 mutants expressing either mCherry-PEX26TM and GFP-PTS1, which label peroxisome membranes and matrices, respectively, or GFP-ATG8a, which labels autophagosomes. I also outline my site-directed mutagenesis experiments designed to further probe the multiple functions of LON2. Finally, I describe experiments demonstrating that various chemicals implicated in modulating autophagy are unable to suppress lon2 defects, suggesting that these chemicals do not inhibit lon2-related autophagy in Arabidopsis and providing a framework for future chemical-genetic screens for autophagy disruption.

4.2. Characterization of a lon2 allelic series

Several lon2 alleles have been isolated by the Bartel lab from various forward-genetic screens for peroxisomal defects (Fig. 4.1A; Table 4.1), but the phenotypes of these mutants had not been directly compared. I designed genotyping primers for several of the lon2 alleles to verify the presence of the reported lesion (Table 2.2). I tested several lon2 alleles for PTS2 processing, and all of the tested alleles displayed the expected PMDH PTS2-processing defect (Fig. 4.1B) that is indicative of impaired matrix protein import.

4.2.1. Missense alleles harboring mutations in protease domain and the arginine finger of the AAA domain of LON2 have differing IBA-resistance and immunoblot phenotypes

Considering the different phenotypes observed when either the Walker A motif of the AAA domain or the active serine of the protease domain is mutated (Goto-Yamada et al., 2014), I compared the IBA resistance, immunoblot phenotypes, and GFP-PTS1 localization of two lon2 missense alleles to a lon2 null allele. I chose lon2-5, lon2-8, and lon2-9 (Fig. 4.1A) for these studies. lon2-5 is the only missense allele in the Bartel lab collection that carries a mutation in the protease domain. The mutation in lon2-5 changes a glycine (G809) between the active resides in the catalytic dyad (S783 and K826) and thus might disrupt the protease activity of
LON2. *lon2-9* (Rinaldi et al., 2016) is a missense allele that carries a mutation that alters the conserved arginine reside (R537K) of the arginine finger motif that is important for intersubunit interaction between the AAA domains of neighboring monomers in the hexameric complex (reviewed in Iyer et al., 2004). I compared these two alleles to *lon2-8*, a likely null allele that carries a nonsense mutation (Q102Stop) in the N-terminal domain (Rinaldi et al., 2016) and has been backcrossed several times.

I measured the lateral root formation of *lon2-5*, *lon2-8*, and *lon2-9* in response to IBA treatment. The protease mutant *lon2-5* and the null mutant *lon2-8* displayed strong IBA resistance whereas the arginine finger mutant *lon2-9* displayed intermediate IBA resistance when compared to wild type and the *lon2-2 atg2-6* double mutant (Fig. 4.1C). Thus *lon2-9* is the only *lon2* allele with intermediate IBA resistance, and this phenotype was subsequently confirmed (Rinaldi et al., 2016).

I observed different immunoblot phenotypes in *lon2-5* and *lon2-9* compared to the null mutant *lon2-8*. ICL was slightly stabilized in *lon2-5* relative to the other two *lon2* mutants (Fig. 4.1D), which is consistent with a previous study showing the ICL is stabilized when the active site serine reside of LON2 is mutated (Goto-Yamada et al., 2014). However, ICL was more dramatically stabilized in the *lon2-2 atg2-6* double mutant than in any of the *lon2* single mutants (Fig. 4.1D). In contrast to ICL, thiolase was more stable in *lon2-9* than in the other two *lon2* mutants. Thiolase was detected in 8-day-old *lon2-9* seedlings whereas thiolase was only detected in 4-day-old *lon2-5* and *lon2-8* seedlings (Fig. 4.1D). *lon2-5* and *lon2-9* exhibited a similar PMDH PTS2-processing defect that was less severe than the processing defect observed in *lon2-8* (Fig. 4.1D). The peroxisomal photorespiration enzyme hydroxypyruvate reductase (HPR) was present in all of the 4-day-old *lon2* seedlings, indicating that differences in developmental timing were unlikely to account for the differences in ICL and thiolase levels. These experiments were performed on backcrossed lines, but further experiments with additionally backcrossed lines could reduce the possibility that background mutations contribute to the observed phenotypes.
Figure 4.1 Characterization of a lon2 allelic series.

(A) Diagram of the LON2 gene. Boxes and lines represent protein coding regions and introns, respectively. The N-terminal domain (tan); AAA domain (blue) including the Walker A motif, the Walker B motif, and arginine finger motif (cyan); protease domain (lavender) including the active serine and lysine (purple) comprising the catalytic dyad; and the C-terminal peroxisomal targeting signal (PTS1) are indicated. The positions of EMS-derived lon2 mutations are indicated by arrows, and T-DNA insertion alleles are indicated by triangles. aa, amino acids. bp, base pairs.

(B) Extracts from 6-day-old seedlings were processed for immunoblotting with antibodies to the indicated proteins.

(C) Lateral root density of 8-day-old wild type (wt), lon2-5, lon2-8, lon2-9, and lon2-2 atg2-6 seedlings grown without or with IBA. Error bars show standard deviations (n = 12).

(D) Extracts from 4-, 6-, and 8-day-old seedlings were processed for immunoblotting with antibodies to the indicated proteins. An asterisk indicates a protein cross-reacting with the ICL antibody.
### Table 4.1 *lon2* alleles available in the Bartel lab.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Alias</th>
<th>Nucleotide change</th>
<th>Protein or transcript change</th>
<th>Screen</th>
<th>Reference</th>
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<td>SALK_043857</td>
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<td>T-DNA</td>
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<td>GABI_034C09</td>
<td>N/A</td>
<td>T-DNA</td>
<td>N/A</td>
<td>Lingard and Bartel, 2009</td>
</tr>
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<td><em>lon2</em>-4</td>
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<td>g829a</td>
<td>Splice site</td>
<td>IBA resistance and sucrose dependence</td>
<td>Farmer et al., 2013</td>
</tr>
<tr>
<td><em>lon2</em>-5</td>
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<td>g4748a</td>
<td>G809E</td>
<td>IBA resistance and sucrose dependence</td>
<td>Woodward and Bartel, unpublished</td>
</tr>
<tr>
<td><em>lon2</em>-6</td>
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<td>g2810a</td>
<td>G445R</td>
<td>GFP-ICL stabilization</td>
<td>Burkhart et al., 2013</td>
</tr>
<tr>
<td><em>lon2</em>-8</td>
<td>R109</td>
<td>c650t</td>
<td>Q102Stop</td>
<td>GFP-PTS1 mislocalization</td>
<td>Rinaldi et al., 2016</td>
</tr>
<tr>
<td><em>lon2</em>-9</td>
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<td>g3611a</td>
<td>R537K</td>
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<td>Fleming, 2016</td>
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<td>g832a</td>
<td>G110E</td>
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<td>Fleming, 2016</td>
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4.2.2. Missense alleles harboring mutations in the arginine finger and in the protease domain of LON2 have similar confocal phenotypes

I observed similar phenotypes in lon2-5, lon2-8, and lon2-9 seedlings expressing GFP-PTS1 using confocal microscopy. lon2 mutants exhibit enlarged peroxisomes (Rinaldi et al., 2016), and this phenotype is suppressed in lon2 mutants when autophagosomes are disrupted (Farmer et al., 2013) or when expressing a protease-inactive lon2 (Goto-Yamada et al., 2014). lon2-5, lon2-8, and lon2-9 all displayed enlarged peroxisomes and cytosolic fluorescence in cotyledon epidermal cells (Fig. 4.2A), cytosolic fluorescence with few puncta in roots (Fig. 4.2B) and numerous small puncta in root tips (Fig. 4.2C), which is consistent with previous reports of lon2 GFP-PTS1 localization (Goto-Yamada et al., 2014; Rinaldi et al., 2016). As expected, wild-type and lon2-2 atg2-6 seedlings displayed small puncta without cytosolic fluorescence in all tissues (Fig. 4.2).

4.3. The large puncta in lon2 mutants may correspond to pexophagy intermediates

As the large GFP-PTS1 peroxisome phenotype in lon2 mutants is suppressed when autophagy is impaired (Fig. 4.2A; Farmer et al., 2013), these structures may be pexophagy intermediates. To explore the identity of these structures, I examined lon2 mutants expressing different fluorophores using confocal microscopy.

4.3.1. The large GFP-PTS1 puncta in lon2-8 co-localize with a peroxisomal membrane marker

I crossed lon2-8 expressing GFP-PTS1 to a line expressing mCherry fused to the transmembrane domain of PEX26 (mCherry-PEX26TM) that I developed to mark peroxisome membranes (see Chapter 5 for more details on mCherry-PEX26TM). GFP and mCherry can be used in conjunction to monitor autophagic flux (Pankiv et al., 2007) because they have different pKa values (~6.0 and 4.5, respectively) and only fluoresce at pH values above their respective pKa (Shaner et al., 2005). The vacuole has a pH of ~4.7 (Shen et al., 2013), so vacuolar GFP is quenched while mCherry continues to fluoresce. Using GFP-PTS1 and mCherry-PEX26TM, any puncta with green and red fluorescence correspond to peroxisomes in the cytosol whereas puncta with only red fluorescence may correspond to peroxisomes that have undergone pexophagy.
Figure 4.2 *lon2*-5, *lon2*-8, and *lon2*-9 display cytosolic localization and large puncta of GFP-PTS1.

(A) Cotyledon epidermal cells, (B) middle root cells, and (C) root tips in 8-day-old wild-type (wt), *lon2*-5, *lon2*-8, *lon2*-9, and *lon2*-2 *atg2*-6 seedlings expressing the peroxisomal matrix marker 35S:GFP-PTS1 were imaged for GFP fluorescence using confocal microscopy. Scale bars = 20 μm.
I examined 6-day-old *lon2-8* seedlings expressing both GFP-PTS1 and mCherry-PEX26TM in cotyledon epidermal cells (Fig. 4.3A), mesophyll cells (Fig. 4.3B), and hypocotyl cells (Fig. 4.3C). Although wild-type controls were not imaged in this experiment, GFP-PTS1 was observed as large puncta and diffuse cytosolic fluorescence typical of *lon2* mutants. mCherry-PEX26 was observed as both rings surrounding GFP-PTS1 characteristic of a peroxisomal membrane marker (Fig. 4.3A, B) and as puncta co-localizing with GFP-PTS1 (Fig. 4.3) that may be representative of condensed peroxisomes. I also observed puncta with red-only fluorescence in hypocotyl cells (Fig. 4.3C), which may correspond to peroxisomes that have undergone pexophagy.

### 4.3.2. Large structures are apparent in *lon2-2* expressing the autophagosomal marker GFP-ATG8a

I examined 6-day-old *lon2-2* seedlings expressing the autophagosome marker GFP-ATG8a (Phillips et al., 2008). If the large GFP-PTS1 puncta observed in *lon2* mutants are pexophagy intermediates, then I expected that similar large puncta would be observed in *lon2-2* expressing GFP-ATG8a. In wild-type seedlings, GFP-ATG8a appeared mostly cytosolic with large, somewhat diffuse orbs that likely correspond to nuclear localization and occasional small puncta that likely correspond to autophagosomes (Fig. 4.4A). In *lon2-2* seedlings, GFP-ATG8a localization was mostly cytosolic with some possible nuclear localization and occasional large, spherical structures (Fig. 4.4B). Some of these structures appeared hollow whereas some appeared solid, and these structures were observed in *lon2-2* cotyledons and hypocotyls (Fig. 4.4B). These structures were similar in size to the large GFP-PTS1 puncta observed in *lon2* mutants (Fig. 4.2A, Fig. 4.3) and were absent in wild-type seedlings, which is consistent with the interpretation that these structures correspond to autophagosomes containing peroxisomes.

### 4.4. Site-directed mutagenesis of LON2

My comparison of *lon2* alleles (Fig. 4.1C, D; Fig. 4.2) and site-directed mutagenesis studies generating AAA-inactive and protease-inactive lon2 variants (Goto-Yamada et al., 2014) indicate that the defects present in *lon2* mutants are indicative of multiple distinct functions of LON2, but these results do not fully explain the reasons for the different *lon2* defects. To more directly probe the functions of LON2, I generated a wild-type LON2 construct and five mutated
Figure 4.3 Examination of *lon2*-8 expressing both a peroxisomal matrix reporter and a peroxisomal membrane reporter.

(A) Cotyledon epidermal cells, (B) cotyledon mesophyll cells, and (C) hypocotyl cells in 6-day-old *lon2*-8 seedlings expressing the peroxisomal matrix marker 35S:GFP-PTS1 (green) and the peroxisomal membrane marker *pUBQ10:mCherry-PEX26TM* were imaged for GFP (green) and mCherry (red) fluorescence using confocal microscopy. White arrows indicate some of the peroxisomes with mCherry-PEX26TM rings that surround GFP-PTS1. Yellow arrows indicate some of the peroxisomes with mCherry-PEX26TM puncta that co-localize with GFP-PTS1. Blue arrows indicate the mCherry-PEX26TM puncta that do not co-localize with GFP-PTS1 and may correspond to peroxisomes that have undergone pexophagy. Scale bars = 20 µm.
Figure 4.4 Aberrant localization of GFP-ATG8a in lon2-2.

Cotyledon epidermal cells (left two columns) and hypocotyl cells (third column) in 6-day-old (A) wild type and (B) lon2-2 seedlings expressing the autophagosome marker 35S:GFP-ATG8a were imaged for GFP fluorescence using confocal microscopy. Blue arrows indicate rings of GFP-ATG8a that may correspond to peroxisomes engulfed by autophagosomes that were observed in lon2-2 but not in wild type. Magenta arrows indicate puncta of GFP-ATG8a that are distinctly brighter and more defined than the unmarked, more diffuse, oblong orbs in wild type. Scale bars = 20 µm.
lon2 constructs (Fig. 4.5). I mutated the Walker A motif of the AAA domain (K414A), the axial pore loop of the AAA domain (Y450A), the Walker B motif of the AAA domain (E476Q), and the protease domain (S783A). I also made a truncated version of lon2 that lacks the N-terminal domain. The Walker A (K414A) and protease (S783A) mutants complement only a subset of lon2 null phenotypes (Goto-Yamada et al., 2014), but not all lon2 phenotypes were reported. A mutation in the axial pore loop, which prevents substrates from moving through the axial pore of the protease, of a bacterial Lon fails to complement susceptibility to proteotoxic shock of a null mutant (Wohlever et al., 2014), suggesting that the axial pore loop is important for the chaperone function of bacterial Lon, and a similarly mutated lon2 may likewise only complement some phenotypes of a lon2 null mutant. Mutating the active glutamate residue to a glutamine in the Walker B motif of bacterial Lon allows the protease to bind but not hydrolyze ATP, turning Lon into a substrate trap (Wohlever et al., 2014). I created a similar mutation in LON2 to generate a substrate-trap lon2 (lon2ST) and also mutated the active serine in the protease domain to ensure that the substrates are not degraded.

I subcloned all of the LON2 variants into destination vectors containing either N-terminal HA or N-terminal GFP. GFP-LON2 localizes to peroxisomes (Goto-Yamada et al., 2014), but whether this construct complements lon2 mutants was not reported. As the N-terminal domain is important for oligomerization in yeast mitochondrial Lon (Kereiče et al., 2016), attaching GFP to the N-terminus may disrupt LON2 oligomerization, so I also generated constructs with the smaller N-terminal HA tag. I did not use C-terminal HA or GFP as LON2 contains a C-terminal PTS1 that is important for peroxisomal localization (Lingard and Bartel, 2009). I transformed the null mutant lon2-8 and selected multiple transformants of each LON2 variant by germinating T1 seeds on Basta (Table 4.2). Once isolated, homozygous lines expressing the lon2 variant proteins will be used to elucidate LON2 function and substrates as described in section 4.6.
Figure 4.5 LON2 constructs from site-directed mutagenesis.
Table 4.2 *LON2* variants transformed into *lon2*-8.

<table>
<thead>
<tr>
<th>Line</th>
<th>Mutation</th>
<th>Number of Basta-resistant T&lt;sub&gt;1&lt;/sub&gt; seedlings</th>
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<td><em>lon2</em>-8 HA-LON2</td>
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</tr>
<tr>
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<td>Walker A</td>
<td>3</td>
</tr>
<tr>
<td><em>lon2</em>-8 HA-lon2&lt;sup&gt;Y450A&lt;/sup&gt;</td>
<td>Axial pore</td>
<td>6</td>
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<tr>
<td><em>lon2</em>-8 HA-lon2&lt;sup&gt;S783A&lt;/sup&gt;</td>
<td>Protease</td>
<td>11</td>
</tr>
<tr>
<td><em>lon2</em>-8 HA-lon2&lt;sup&gt;274-888&lt;/sup&gt;</td>
<td>Truncation</td>
<td>9</td>
</tr>
<tr>
<td><em>lon2</em>-8 HA-lon2&lt;sup&gt;ST&lt;/sup&gt;</td>
<td>Substrate trap</td>
<td>4</td>
</tr>
<tr>
<td><em>lon2</em>-8 eGFP-LON2</td>
<td>Wild type</td>
<td>18</td>
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<td>Walker A</td>
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</tr>
<tr>
<td><em>lon2</em>-8 eGFP-lon2&lt;sup&gt;Y450A&lt;/sup&gt;</td>
<td>Axial pore</td>
<td>12</td>
</tr>
<tr>
<td><em>lon2</em>-8 eGFP-lon2&lt;sup&gt;S783A&lt;/sup&gt;</td>
<td>Protease</td>
<td>3</td>
</tr>
<tr>
<td><em>lon2</em>-8 eGFP-lon2&lt;sup&gt;274-888&lt;/sup&gt;</td>
<td>Truncation</td>
<td>3</td>
</tr>
<tr>
<td><em>lon2</em>-8 eGFP-lon2&lt;sup&gt;ST&lt;/sup&gt;</td>
<td>Substrate trap</td>
<td>10</td>
</tr>
</tbody>
</table>
4.5. Chemical autophagy inhibitors did not suppress lon2 defects

In addition to genetic disruption of autophagy, chemical autophagy inhibitors are invaluable tools for studying autophagy. Chemical autophagy inhibitors could potentially expedite studies of autophagy in various Arabidopsis mutants by circumventing the requirement of crossing the mutant of interest to an atg mutant. Numerous chemical autophagy inhibitors are frequently used in yeast and mammalian studies, but the ability of these inhibitors to suppress lon2 defects has not been reported. Moreover, lon2 mutants serve as a platform useful for studying the efficacy of purported chemical autophagy inhibitors in Arabidopsis and for identifying novel chemical autophagy inhibitors by performing chemical genetics. I tested two chemicals – 3-methyladenine and verteporfin – for the ability to suppress lon2 phenotypes and did not observe any suppression, indicating that these chemicals do not inhibit autophagy in Arabidopsis. With the assistance of a rotating graduate student, I conducted a pilot chemical genetics screen for potential chemical autophagy inhibitors but did not recover any chemical autophagy inhibitors.

4.5.1. 3-methyladenine did not suppress lon2 defects

3-methyladenine (3-MA) is commonly used as an autophagy inhibitor that acts by blocking phosphatidylinositol 3-kinase activity, causing pleiotropic effects in addition to autophagy inhibition (reviewed in Klionsky et al., 2016). 3-MA inhibits autophagy in plants (reviewed in Bassham, 2007), so I tested the ability of 3-MA to suppress lon2-2 defects (Fig. 4.6). I measured IBA responsiveness of wild-type and lon2-2 seedlings treated with 0.625 mM, 3 mM, or 5 mM 3-MA. 3-MA did not restore IBA sensitivity of lon2-2 in terms of root length (Fig. 4.6A) or lateral root production (Fig. 4.6B). Moreover, root length decreased with increasing concentration of 3-MA in both wild-type and lon2-2 (Fig. 4.6A), suggesting that 3-MA is generally toxic to seedlings at high concentrations. Interestingly, wild-type roots were somewhat longer when grown on 0.625 mM 3-MA and IBA than with just IBA (Fig. 4.6A), indicating that 3-MA might cause IBA resistance in wild-type seedlings, which is not easily explained by an inhibitory effect on autophagy. Lateral root production of wild-type seedlings grown on IBA decreased with increasing concentration of 3-MA (Fig. 4.6B), but this decrease is likely due to
the toxic effect of high concentrations of 3-MA on seedling growth. I did not observe any restoration of lon2-2 PMDH PTS2-processing by 3-MA (Fig. 4.6C). Together, these results suggest that chronic 3-MA treatment does not inhibit autophagy of lon2-2 peroxisomes.

4.5.2. Verteporfin did not suppress lon2 defects

Verteporfin was identified as a potential autophagy inhibitor in a screen for chemicals inhibiting autophagosome formation in mammalian cells (Donohue et al., 2011), but the mechanism of action of this chemical is unknown. To see if verteporfin disrupts autophagy in Arabidopsis, I tested the ability of verteporfin to inhibit lon2-2 defects (Fig. 4.7). Rather than restoring IBA sensitivity to lon2-2, verteporfin caused slight IBA or 2,4-dichlorophenoxybutyric acid resistance in wild-type seedlings in terms of light-grown root length (Fig. 4.7A) and dark-grown hypocotyl length (Fig. 4.7C), respectively. Similarly, verteporfin rendered lateral root production resistance to the stimulatory effects of IBA in wild type (Fig. 4.7B). I did not observe any effect on verteporfin on PEX14 levels, thiolase levels, or PMDH PTS2 processing at any tested concentration in wild-type or lon2-2 seedlings (Fig. 4.7D).

I tested the effect of verteporfin on atg7-3 and nbr1-1 to ascertain the importance of autophagy in the verteporfin-induced IBA resistance observed in wild type, and I also treated the seedlings with the synthetic auxin 1-naphtaleneacetic acid (NAA) to see if the effect of verteporfin was altering auxin responsiveness in general. If verteporfin induces IBA resistance in wild-type by promoting pexophagy, then I expected that verteporfin would not induce IBA resistance in atg7-3. I found that Verteporfin conferred IBA resistance in wild-type, atg7-3, and nbr1-1 seedlings in terms of root length (Fig. 4.7E) and lateral root production (Fig. 4.7F), suggesting autophagy is not necessary for the effect of verteporfin. Verteporfin also induced slight NAA resistance in wild-type seedlings in terms of root length (Fig. 4.7E) and lateral root production (Fig. 4.7F), suggesting that verteporfin inhibits auxin responsiveness, but the effect was not as strong as that observed for IBA. Taken together, these results indicate that verteporfin does not inhibit autophagy of peroxisomes under the tested conditions but may inhibit auxin responsiveness in plants in an autophagy-independent manner.
Figure 4.6 The autophagy inhibitor 3-methyladenine does not suppress lon2-2 defects. 
(A) Root length and (B) lateral root density of 6-day-old wild-type (wt) and lon2-2 seedlings grown on varying concentrations of 3-methyladenine (3-MA) without or with IBA. Points indicate the mean value (n=12). 
(C) Extracts from 6-day-old seedlings measured in panels A and B were processed for immunoblotting with an antibody to PMDH.
Figure 4.7 Analysis of the effect of verteporfin on auxin responsiveness.

(A) Root length and (B) lateral root density of 8-day-old wild-type (wt) and lon2-2 seedlings grown on varying concentrations of verteporfin without or with IBA. Points indicate the mean value (n≥5).

(C) Dark-grown hypocotyl length of 8-day-old wild-type (wt) and lon2-2 seedlings grown on varying concentrations of verteporfin without or with 2,4-dichlorophenoxybutyric acid (2,4-DB). Points indicate the mean value (n≥5).

(D) Extracts from 8-day-old seedlings measured in panels A and B were processed for immunoblotting with antibodies to the indicated proteins.

(E) Root length and (F) lateral root density of 8-day-old wild-type (wt), atg7-3, and nbr1-1 seedlings grown without or with verteporfin, IBA, and/or 1-napthaleneacetic acid NAA. Error bars show standard deviations (n≥9).
4.5.3. Screening for lon2-suppressing chemicals did not reveal any autophagy-inhibiting chemicals

I mentored rotating graduate student Roxanna Llinas in a chemical screen of part of the Microsource Spectrum collection library for potential autophagy inhibitors for plants by screening for chemicals that suppress the IBA resistance of lon2-2 lateral root formation. Eight potential hits emerged from Roxanna’s screen of 400 compounds: adenosine, bisacodyl, R-(-)-apomorphine hydrochloride hemihydrate, thioridazine hydrochloride, promazine hydrochloride, metoclopramide hydrochloride, meclofenamic acid, and prochlorperazine dimaleate. I retested these chemicals for the ability to suppress IBA resistance in lon2-2. I grew wild-type and lon2-2 seedlings on each of the chemicals at 25 µM with or without IBA (Fig. 4.8A, B). Of these chemicals, only prochlorperazine dimaleate stimulated lateral root production in lon2-2 in response to IBA (Fig. 4.8B) although root length was severely stunted (Fig. 4.8A). I further tested prochlorperazine dimaleate at several lower concentrations ranging from 5 µM to 25 µM to see if prochlorperazine dimaleate stimulated lateral root production in lon2-2 in a dose-dependent manner. Root length decreased in wild-type and lon2-2 seedlings with increasing concentration of prochlorperazine dimaleate (Fig. 4.8C), indicating a toxic effect of the chemical on seedling growth. Moreover, prochlorperazine dimaleate did not have a pronounced effect on stimulating lateral root production in response to IBA (Fig. 4.8D); although the number of lateral roots per millimeter of root length increased somewhat in both wild-type and lon2-2 with increasing concentration of prochlorperazine dimaleate, the total number of lateral roots did not increase. The negative effect of prochlorperazine dimaleate on seedling growth precludes any usefulness that the chemical may have as an autophagy inhibitor in plants.
Figure 4.8 Retesting potential autophagy inhibitor chemicals emerging from a screen for lon2-2-suppressing chemicals.

(A) Root length and (B) lateral root density of 9-day-old wild-type (wt) and lon2-2 seedlings grown on 25 µM of the indicated chemical without or with IBA. An abbreviations key for the chemicals is shown on the lower right. Error bars show standard deviations (n≥6).

(C) Root length and (D) lateral root density of 8-day-old wild-type (wt) and lon2-2 seedlings grown on varying concentrations of prochlorperazine dimaleate without or with IBA. Points indicate the mean value (n≥6).
4.6. Conclusions and discussion

The characterization of a *lon2* allelic series that I performed provides some insight into the complex functions of LON2. The intermediate IBA resistance (Fig. 4.1C) and PMDH PTS2-processing defect (Fig. 4.1B, D) of *lon2*-9 suggest that LON2 function is only partially impaired, but the confocal phenotype of *lon2*-9 (Fig. 4.2) suggests that at least some functionality of LON2 is severely impaired. Similarly, *lon2*-5 displayed strong IBA resistance (Fig. 4.1C) and a confocal phenotype similar to the null *lon2*-8 allele (Fig. 4.2) but displayed a weaker PMDH PTS2-processing defect than *lon2*-8 (Fig. 4.1D), implying that the *lon2*-5 protein retains some function. Moreover, ICL is slightly stabilized in *lon2*-5, but thiolase is more stable in *lon2*-9 than in either of the other *lon2* mutants (Fig. 4.1D), hinting that LON2 interacts with various substrates in different ways.

*lon2*-5 *atg2*-4, *lon2*-8 *atg2*-4, and *lon2*-9 *atg2*-4 doubles mutants may be informative of the role of pexophagy in the different *lon2* alleles. Although many *lon2*-2 defects, such as IBA resistance and a PTS2 processing defect, are suppressed by *atg* mutants, *lon2*-2 *atg* double mutants stabilize glyoxylate cycle enzymes and thiolase (Farmer et al., 2013). If the protease activity of *lon2*-5 or *lon2*-9 mutant protein is not compromised, the respective *lon2* *atg2*-4 double mutant might not stabilize certain peroxisomal proteins such as the glyoxylate cycle enzymes. Interestingly, *lon2* mutants and *atg* mutants both accumulate electron-dense inclusions of catalase in peroxisomes (Shibata et al., 2013; Goto-Yamada et al., 2014), indicating roles for both LON2 and autophagy in maintaining peroxisome quality, but the significance of the accumulated catalase for peroxisome (dys)function has yet to be determined. Analysis of catalase levels and function in the *lon2* *atg2*-4 double mutants might also be informative. Other defects in *lon2*-2 *atg* double mutants may yet be discovered.

Although several of these alleles were recovered from screens that included sucrose dependence as a criterion (*lon2*-4, *lon2*-5, *lon2*-277, and *lon2*-310) (Fleming, 2016), *lon2* mutants do not present strong sucrose dependence (Lingard and Bartel, 2009; Burkhart et al., 2013; Rinaldi et al., 2016). Moreover, *lon2*-6 was recovered from a screen for stabilized GFP-ICL but did not retest for GFP-ICL stabilization (Burkhart et al., 2013). Throughout our studies using *lon2* mutants, I and others in the Bartel lab have noted that *lon2* mutants display mild germination defects that worsen as seeds age especially if kept at room temperature, but we have not conducted any formal experiments to quantify these observations. It is possible that the *lon2*
alleles recovered from screens for sucrose dependence or GFP-ICL stabilization appeared to be sucrose dependent or to stabilize GFP-ICL in the primary screen due to delayed germination.

In addition to lon2-5 and lon2-9, two other lon2 missense alleles have been isolated in the Bartel lab: lon2-6 and lon2-310, which carry mutations altering G445 to an arginine and G110 to a glutamate, respectively (Fig. 4.1A, Table 4.1). Neither lon2-6 nor lon2-310 has a reported phenotype different from a null lon2 allele (Burkhart et al., 2013; Fleming, 2016). We do not have an antibody that recognizes LON2, so it is unknown whether any of the missense mutations in LON2 destabilize the resulting lon2 protein.

The large puncta that appear in lon2 mutants expressing peroxisomally-targeted GFP (Fig. 4.2) remain mysterious. As atg mutants suppress this phenotype in lon2 mutants, it is possible that these large puncta correspond to peroxisomes that are undergoing pexophagy. I have two hypotheses to explain the large puncta. One hypothesis is that the large puncta correspond to peroxisomes that are inside an autophagosome. The other hypothesis is that the large puncta correspond to peroxisomes that are inside compartments known as autolysosomes, which are a fusion of endosomes, autophagosomes, and vesicles from the vacuole (Oh-ye et al., 2011); these autolysosomes may be specific to plants and allow cells to begin degrading substrates of autophagy before the substrate is sent to the vacuole.

The confocal experiments that I performed provide some clues to the identity of the large puncta present in lon2 mutants. Examination of lon2-8 expressing new reporters revealed that mCherry-PEX26TM peroxisome membrane marker co-localized with GFP-PTS1 most of the time, indicating that most of the large puncta are not inside the vacuole (where GFP fluorescence is quenched; Pankiv et al., 2007). Several puncta with only red fluorescence were visible in hypocotyl cells, suggesting that pexophagy might be higher in hypocotyls relative to cotyledons, which is consistent with previous reports (Kim et al., 2013). Interestingly, the mCherry-PEX26 sometimes appeared as rings and sometimes appeared as puncta (Fig. 4.3). The ring pattern is consistent with mCherry-PEX26TM acting as a peroxisomal membrane marker, and the mCherry puncta may be indicative of disrupted or condensed peroxisome membranes of peroxisomes that are being degraded. Visualization of wild-type and atg seedlings expressing GFP-PTS1 and mCherry-PEX26TM may be helpful for confirming these structures; if the red-only puncta in the hypocotyls correspond to peroxisomes that have undergone pexophagy, the red-only puncta would be expected to be present in wild-type seedlings but absent in atg mutants. Visualization
of lon2-2 expressing GFP-ATG8a revealed structures consistent with the large GFP-PTS1 puncta in lon2 corresponding to pexophagy intermediates. Large rings of GFP-ATG8a appeared in lon2-2 but not wild-type seedlings (Fig. 4.4), and as GFP-ATG8a marks autophagosome membranes, these rings may correspond to engulfed peroxisomes. Interestingly, sometimes large puncta were observed in lon2-2 instead of rings, which is reminiscent of the mCherry-PEX26TM observed in lon2-8 seedlings (Fig. 4.3); these puncta may correspond to peroxisomes that are undergoing degradation. Co-localization studies using GFP-ATG8a in conjunction with a peroxisomal marker, such as mCherry-PEX26TM or mRuby-PTS1, will be needed to determine whether these rings correspond to peroxisomes undergoing pexophagy. Treatment of lon2 seedlings expressing GFP-ATG8a and a peroxisomal reporter with concanamycin A, which blocks vacuolar degradation, may also be informative; if pexophagy is higher in lon2 than in wild type, then the number of vacuolar peroxisomes co-localizing with ATG8a would be expected to be higher in lon2 than in wild type. Autolysosomes can be visualized using the endocytosis tracker FM4-64 and the cysteine protease inhibitor E-64d (Oh-ye et al., 2011), and visualization of lon2 mutants expressing GFP-PTS1 in combination with FM4-64 and E-64d may indicate if the large puncta in lon2 correspond to autolysosomes.

The lon2 variants that I generated by site-directed mutagenesis (Fig. 4.5) will be useful for probing the functions of LON2. Once homozygous lines of the lon2 variants in the lon2-8 null allele have been isolated, the suite of lon2 phenotypes can be examined: IBA resistance in lateral root formation, PTS2 processing defect, thiolase destabilization, ICL or MLS stabilization, large puncta, and hypersensitivity to dark treatment. I expect that different lon2 variants will rescue different phenotypes of lon2-8.

The substrate-trapping lon2 variant (lon2ST) may be useful for identification of LON2 substrates. I hypothesize that accumulation of a certain substrate in the absence of LON2 acts as a signal for pexophagy. By identifying the substrates of LON2, the signal(s) for pexophagy can be uncovered, and the regulatory role of LON2 can be better understood. The lon2ST transgene can be crossed into a lon2-8 atg2-4 double mutant, which will prevent the substrates from being degraded by pexophagy. Pull down of GFP-lon2ST or HA-lon2ST from plant extracts could be performed, and the differences between the lon2-8 and lon2-8 atg2-4 mutants could be compared to identify substrates that accumulate in lon2-8 atg2-4. The interacting substrates might be identified using mass spectrometry, and comparison of the results to the AraPerox database.
(Reumann et al., 2004) may facilitate identification of the proteins. Immunoblotting can also be used to test potential substrates for which we have antibodies (e.g., ICL). Interactions may be verified using yeast two-hybrid experiments (for non-modified proteins), and reciprocal co-IPs may be possible when antibodies are available. As LON2 is positioned to be involved in quality control, I expect that many of its substrates may exhibit oxidative damage, which will be accompanied by carbonylation. Moreover, particular attention to kinases may be informative as the kinase PINK1 is involved in mitophagy (Narendra et al., 2010; Vives-Bauza et al., 2010; Kane et al., 2014; Kazlauskaite et al., 2014; Koyano et al., 2014). Once candidate substrates have been identified, insertional alleles of the candidate substrates could be used to test their role in pexophagy.

lon2 mutants provide an excellent platform for studying the efficacy of chemical autophagy inhibitors on Arabidopsis. I observed no suppression of lon2-2 defects by 3-MA (Fig. 4.6), which was not unexpected as 3-MA is a phosphatidylinositol 3-kinase inhibitor that is often used as an autophagy inhibitor but that induces autophagy with prolonged treatment (reviewed in Klionsky et al., 2016). Bay11-7082 recently emerged from a chemical screen for chemicals that alter the rate of decay of the peroxisomal enzyme luciferase in S. cerevisiae, and Bay11-7082 acts as an autophagy inhibitor that blocks autophagy upstream of autophagosome/vacuole fusion in yeast, mammalian cells, and lace plant (Aponogeton madagascariensis) (Mishra et al., 2017). It would be interesting to test the ability of Bay11-7082 to suppress Arabidopsis lon2 mutants. Although our pilot chemical screen was not successful (Fig. 4.8), screening for chemicals from other libraries that promote lon2 lateral root formation on IBA may uncover autophagy or pexophagy inhibitors. The chemicals identified as lon2 suppressors might be more specific inhibitors than the phosphatidylinositol 3-kinase inhibitors, such as 3-MA, that are often used as autophagy inhibitors. The chemicals identified from a lon2 chemical suppressor screen would be expected to act upstream of autophagosome closure; if the chemical inhibited autophagy in a step downstream of autophagosome closure, then peroxisomes would still be sequestered and thus unable to import matrix proteins and substrates such as IBA.
Chapter 5: Generation of a tandem-fluorescent peroxisomal membrane marker for monitoring pexophagy

5.1. Introduction

Recent research provides evidence for pexophagy in plants (Farmer et al., 2013; Kim et al., 2013; Shibata et al., 2013), but an assay to directly monitor pexophagy is needed to further investigate this pathway. One potential assay utilizes a tandem-fluorescent reporter comprised of GFP and mCherry linked to LC3 to monitor autophagic flux (Pankiv et al., 2007), and substitution of the LC3 moiety with the transmembrane domain of PMP PEX26 to this tandem-fluorescent reporter allows monitoring of pexophagic flux in mammalian cells (Deosaran et al., 2013). GFP and mCherry have different pKa values (~6.0 and 4.5, respectively) and only fluoresce at pH values above their respective pKa (Shaner et al., 2005). The vacuole has a pH of ~4.7 (Shen et al., 2013), so vacuolar GFP is quenched while mCherry continues to fluoresce. Using confocal microscopy, puncta with co-localized GFP and mCherry correspond to peroxisomes in the cytosol; mCherry puncta that do not co-localize with GFP correspond to vacuolar peroxisomes that have undergone pexophagy (Deosaran et al., 2013).

This tandem-fluorescent construct can be adapted for other purposes. Expression of a ubiquitinated PMP stimulates pexophagy in mammalian cells (Kim et al., 2008), but ubiquitination has not been directly implicated in pexophagy in plants. Addition of a non-cleavable ubiquitin moiety to the tandem-fluorescent construct provides a method to test the sufficiency of ubiquitin to stimulate plant pexophagy. Removal of one of the fluorophores in the tandem-fluorescent construct results in a peroxisomal membrane marker, which could be useful for probing peroxisome function and morphology in a variety of mutant backgrounds.

5.2. Generation of fluorescent peroxisomal membrane reporters

We ordered a synthesized, codon-optimized gene from GenScript that encodes the tandem-fluorescent peroxisome marker comprised of a non-cleavable ubiquitin (C-terminal G76V), GFP, mCherry, and the transmembrane domain of PEX26 (PEX26TM) (Fig. 5.1). I included restriction sites to allow removal of ubiquitin, GFP, mCherry, and/or the
**Figure 5.1** Constructs for labeling peroxisomal membranes and monitoring pexophagy.

(A) Ubiquitin-GFP-mCherry-PEX26TM (UGMT) was designed to test the sufficiency of ubiquitin to stimulate pexophagy. The C-terminal transmembrane region of PEX26 (PEX26TM) will allow the gene product to be inserted into the peroxisomal membrane with the remaining components facing the cytosol. GFP and mCherry both fluoresce at cytosolic pH, but only mCherry fluoresces at vacuolar pH, allowing distinction between peroxisomes in the cytosol and those that have been sent to the vacuole for degradation via pexophagy. A non-cleavable ubiquitin (Gly76Val) is added to test the sufficiency of ubiquitin to stimulate pexophagy. Restriction sites between components in the synthesized construct allow facile generation of various drop-out constructs. Constructs were subcloned into vectors that drive expression of the reporter protein by either the 35S or the pUBQ10 constitutive promoters.

(B) GFP-mCherry-PEX26TM (GMT) was designed to monitor pexophagy.

(C) mCherry-PEX26TM (MT) was designed to provide a generally useful peroxisome-membrane marker that is compatible with our GFP-PTS1 peroxisome matrix marker.
PEX26TM. I subcloned the gene into the pENTR entry vector, dropped out either the ubiquitin moiety alone to generate the tandem-fluorescent construct for monitoring pexophagy (Fig. 5.1B) or both the ubiquitin and the GFP moiety to generate a peroxisome membrane marker (Fig. 5.1C), and subcloned the constructs into destination vectors that drive expression of the fusion protein by either the 35S or pUBQ10 constitutive promoters. I will hereafter refer to the ubiquitin-GFP-mCherry-PEX26TM construct as UGMT (Fig. 5.1A), the GFP-mCherry-PEX26TM construct as GMT (Fig. 5.1B), and mCherry-PEX26TM construct as MT (Fig. 5.1C).

I transformed wild-type plants with the various constructs, selected transformants (T1 plants) by resistance to the herbicide Basta, tested whether the T1 plants were expressing the transgenes by performing immunoblots, and verified that the T2 plants contained the transgenes by genotyping (Table 2.7).

5.3. Characterization of a ubiquitin-tagged peroxisomal membrane reporter

5.3.1. Ubiquitin-GFP-mCherry-PEX26TM protein is not highly expressed

I recovered nine 35S:UGMT and seven pUBQ10:UGMT transformants. All of these lines were Basta resistant and contained the respective transgene. I performed immunoblot analysis on the T2 lines and did not detect the presence of the UGMT protein, which has a molecular weight of 73.5 kDa, in any of the transformed lines using an antibody that detects mCherry (Fig. 5.2A). I did not detect a PMDH PTS2-processing defect in any of the transformed lines (Fig. 5.2A), indicating that peroxisomal function was not notably impaired. I isolated T3 lines transformed with 35S:UGMT (T3-2C2) and pUBQ10:UGMT (T3-3G2) stably expressing Basta resistance.

I examined 8-day-old pUBQ10:UGMT T3-3G2 seedlings using confocal microscopy. Using our normal settings, I did not detect any GFP or mCherry fluorescence corresponding to peroxisomes. Only when increasing the digital gain did I detect faint puncta, but these puncta were barely brighter than the background autofluorescence (Fig. 5.2B).
Figure 5.2 Ubiquitin-GFP-mCherry-PEX26TM does not accumulate in seedlings containing the transgene.

(A) Extracts from 8-day-old T₂ seedlings containing 35S:UGMT or pUBQ10:UGMT were processed for immunoblotting with antibodies to mCherry (top panel) or to the indicated proteins. Extract from 40-day-old plants expressing 35S:mCherry-PTS1 provide a positive control for the mCherry antibody. Molecular weights (kDa) are indicated on the left. The predicted location of the 73.5-kDa UGMT protein is indicated on the right. An asterisk indicates a protein cross-reacting with the mCherry antibody.

(B) Cotyledon epidermal cells in 8-day-old seedlings expressing pUBQ10:UGMT were imaged for GFP (green) and mCherry (red) fluorescence using confocal microscopy. The gain in this image was increased to reveal faint fluorescence. Scale bar = 20 µm.
5.3.2. Ubiquitin-GFP-mCherry-PEX26TM does not impair peroxisome function

One explanation for low UGMT levels might be that the reporter induces pexophagy, thus leading to UGMT degradation along with the peroxisome. If expression of UGMT induces pexophagy, then I expected that plants expressing UGMT would phenocopy lon2-2. I measured IBA responsiveness in T3 lines stably carrying 35S:UGMT or pUBQ10:UGMT but did not observe IBA resistance in either UGMT-transformed line compared to wild type in terms of root length (Fig. 5.3A) or lateral root development (Fig. 5.3B). I performed immunoblot analysis on 4-, 6-, and 8-day old T3 lines stably carrying 35S:UGMT or pUBQ10:UGMT and did not detect UGMT protein at any time point using an antibody detecting mCherry (Fig. 5.3C). Moreover, unlike in lon2-2, PEX14 and thiolase levels were not lowered (Fig. 5.3C), and I did not detect a PMDH PTS2-processing defect in the UGMT-transformed lines (Fig. 5.2A, Fig. 5.3C). Together, these results suggest that the UGMT transgene does not impair peroxisome function or stimulate pexophagy in plants.

5.4. Confocal microscopy of peroxisomal membrane reporters

5.4.1. mCherry-PEX26TM and GFP-mCherry-PEX26TM appear to localize to peroxisome membranes

I recovered several 35S:MT, pUBQ10:MT, 35S:GMT, and pUBQ10:GMT transformants and performed immunoblot analysis using an antibody that detects mCherry to select lines that accumulated the respective reporter protein. I did not detect the reporter in any of the 35S:GMT T1 plants and did not advance any 35S:GMT lines. I performed immunoblot analysis on the 35S:MT, pUBQ10:MT, and pUBQ10:GMT T2 lines and detected the MT protein in all three lines expressing an MT transgene and detected the GMT protein in two of the six pUBQ10:GMT lines (T2-5G and T2-6B) (Fig. 5.4A). Interestingly, the level of GMT protein was lower than that of MT protein, and the level of both of these proteins was lower than the level of mCherry-PTS1 protein driven by the constitutive 35S promoter (Fig. 5.4A). I also detected a lower molecular weight protein in the 35S:MT and pUBQ10:MT lines that may correspond to proteolytically cleaved MT protein (Fig. 5.4A).

I examined 6-day-old pUBQ10:MT T3-4B seedlings and 7-day-old pUBQ10:GMT T3-6B3 seedlings using confocal microscopy. I frequently observed puncta and occasionally
Figure 5.3 Ubiquitin-GFP-mCherry-PEX26TM does not impair peroxisome function. (A) Root length and (B) lateral root density of 8-day-old seedlings grown without or with IBA. Error bars show standard deviations (n = 10). (C) Extracts from 4-, 6-, and 8-day-old seedlings were processed for immunoblotting with antibodies to mCherry (top panel) or to the indicated proteins. Molecular weights (kDa) are indicated on the left. The predicted location of the 73.5-kDa UGMT protein is indicated on the right. Asterisks indicate proteins cross-reacting with the mCherry antibody.
observed rings in both lines (Fig. 5.4B, C), which is consistent with these reporters labeling peroxisome membranes. The numerous puncta in cotyledon cells, hypocotyl cells, and root cells of pUBQ10:MT T_3-4B seedlings (Fig. 5.5) were approximately the expected size of peroxisomes, consistent with the MT reporter labeling peroxisomes. Moreover, I observed the expected colocalization of GFP and mCherry fluorescence in seedlings expressing GMT (Fig. 5.4C).

5.4.2. The GFP-mCherry-PEX26TM reporter may monitor pexophagic flux

If GMT can monitor pexophagic flux, then I expected to see GFP and mCherry puncta co-localizing in the cytosol, corresponding to peroxisomes in the cytosol, and mCherry-only puncta in the vacuole, corresponding peroxisomes that have undergone pexophagy. Moreover, as the vacuole fills most of the volume of the cell, I expected to see cytosolic (GFP and mCherry) peroxisomes in the periphery of the cell and vacuolar (mCherry only) peroxisomes in the center of the cell. Furthermore, I expected to observe a higher rate of pexophagic flux in hypocotyls relative to other tissues as glyoxylate cycle enzymes are stabilized and GFP-PTS1 puncta are more numerous in hypocotyls of atg mutants (Fig. 3.8C; Kim et al., 2013).

I examined 4-, 5-, 6-, and 7-day-old pUBQ10:GMT T_3-6B3 seedlings using confocal microscopy. In cotyledons, I observed numerous puncta with both GFP and mCherry fluorescence, but I did not observe puncta with only mCherry fluorescence (Fig. 5.6A). In root tips, I observed numerous puncta with both GFP and mCherry fluorescence in all seedlings (Fig. 5.6 B, C) and also observed numerous puncta with only mCherry fluorescence in some seedlings (Fig. 5.6B) but not in other seedlings (Fig. 5.6C). When observed, only one of these mCherry-only puncta in root tips was typically observed per cell (Fig. 5.6A).

I examined hypocotyls in 4-, 5-, 6-, and 7-day-old pUBQ10:GMT T_3-6B3 seedlings and observed numerous puncta with both GFP and mCherry fluorescence at all ages (Fig. 5.7). I also observed puncta with only mCherry fluorescence at all ages (Fig. 5.7B, C, D) but not in every seedling (Fig. 5.7A). In contrast to the puncta with only mCherry fluorescence observed in root tips, the mCherry-only puncta in hypocotyls sometimes appeared in clusters (Fig. 5.7B, C), but sometimes only one of these puncta was present per cell (Fig. 5.7C, D). The puncta with only mCherry fluorescence often appeared in the central region of the cells (Fig. 5.7B, C, D), which is consistent with vacuolar localization, and were larger than the average puncta with both GFP and mCherry fluorescence (Fig. 7B, C, D). Diffuse red vacuolar fluorescence was sometimes
Figure 5.4 GFP-mCherry-PEX26TM and mCherry-PEX26TM label peroxisome membranes. (A) Extracts from 8-day-old seedlings were processed for immunoblotting with antibodies to mCherry (top panel) or to the indicated proteins. Molecular weights (kDa) are indicated on the left. An asterisk indicates a protein cross-reacting with the mCherry antibody.
(B) Cotyledon epidermal cells in 6-day-old seedlings expressing pUBQ10:MT were imaged for mCherry (red) fluorescence using confocal microscopy. White arrows indicate some of the peroxisomes with mCherry-PEX26TM rings. Yellow arrows indicate some of the peroxisomes with mCherry-PEX26TM puncta. Scale bar = 5 µm.

(C) Cotyledon epidermal cells in 7-day-old seedlings expressing pUBQ10:GMT were imaged for GFP (green) and mCherry (red) fluorescence using confocal microscopy. White arrows indicate some of the peroxisomes with GFP-mCherry-PEX26TM rings. Yellow arrows indicate some of the peroxisomes with GFP-mCherry-PEX26TM puncta. Scale bar = 5 µm.
**Figure 5.5** mCherry-PEX26TM is expressed throughout the plant. Cotyledon cells, hypocotyl cells, and root cells in 6-day-old seedlings expressing *pUBQ10:MT* were imaged for mCherry (red) fluorescence using confocal microscopy. Images are Z-stack maximum projections of a total thickness of 26 µm (cotyledon), 23 µm (hypocotyl), and 28 µm (root) at 1.7 µm per slice. Scale bar = 20 µm.
observed (Fig. 5.7B, D), which is consistent with peroxisomes being degraded inside the vacuole.

5.5. Conclusions and discussion

I generated six constructs (three reporters each driven by two different constitutive promoters) to mark peroxisome membranes (Fig. 5.1) and recovered numerous transformants of each. Curiously, I did not detect the presence of the UGMT protein in any of the transformants despite the presence of the transgene and the concomitant Basta resistance used for selection (Fig. 5.2). While the lack of detectable UGMT could be consistent with stimulated pexophagy, lines containing a UGMT transgene did not display any apparent peroxisomal defects (Fig. 5.3), suggesting that the UGMT protein is either being degraded by another mechanism or is not synthesized. I treated seedlings with either the autophagy inhibitor 3-MA or the proteasomal inhibitor MG132 to determine if blocking degradation by either autophagy or the proteasome would stabilize UGMT, but I did not detect any stabilized UGMT upon treatment with either chemical (not shown). However, the results from this experiment were inconclusive and lacked a proper mCherry-expressing line as a control. Moreover, my results using 3-MA on lon2-2 (Fig. 4.6) suggest that 3-MA is not an effective pexophagy inhibitor in plants. Crossing UGMT-expressing lines to atg mutants would provide a more effective means to block autophagy than using 3-MA, and crossing UGMT-expressing lines to an nbr1 mutant could determine if NBR1 is important for degradation of UGMT.

I observed less reporter protein when more moieties were attached to PEX26TM (Fig. 5.4A), hinting that these proteins might be selectively recognized and degraded. Perhaps when more moieties are attached to the PEX26TM, the reporter protein is more easily recognized and removed and degraded. A screen for mutants with restored UGMT protein might recover mutants defective in this degradation pathway. If a larger protein attached to PEX26TM results in degradation, then removal of GFP and/or mCherry from the UGMT construct might yield a ubiquitin-tagged PMP that is capable of stimulating pexophagy in a manner similar to that observed in mammalian cells (Kim et al., 2008). PEX26 contains a single-pass transmembrane domain (Gonzalez et al., 2017), and attachment of ubiquitin-GFP-mCherry to a the transmembrane domain of a PMP with a multi-pass transmembrane domain, such as PXA1 (Zolman et al., 2001), might make removal of the transgenic protein more difficult, stabilizing
Figure 5.6 Examination of GFP-mCherry-PEX26TM in cotyledons and root tips.

(A) Cotyledon cells in 5-day-old seedlings expressing pUBQ10:GMT were imaged for GFP (green) and mCherry (red) fluorescence using confocal microscopy. Images are Z-stack maximum projections of a total thickness of 23 µm at 1.7 µm per slice. Scale bar = 20 µm.

Root tips in (B) 4-day-old and (C) 5-day-old seedlings expressing pUBQ10:GMT were imaged for GFP (green) and mCherry (red) fluorescence using confocal microscopy. Images are Z-stack maximum projections of a total thickness of (B) 28 µm and (C) 23 µm at 1.7 µm per slice. Blue arrows in panel (B) indicate the mCherry puncta that do not co-localize with GFP and may correspond to peroxisomes that have undergone pexophagy. Scale bars = 10 µm.
Hypocotyl cells in (A and B) 4-, (C) 5-, and (D) 7-day-old seedlings expressing \textit{pUBQ10:GMT} were imaged for GFP (green) and mCherry (red) fluorescence using confocal microscopy. Images are Z-stack maximum projections of a total thickness of (A) 12 \( \mu \text{m} \), (B and D) 23 \( \mu \text{m} \), and (C) 17 \( \mu \text{m} \) at 1.7 \( \mu \text{m} \) per slice. Blue and white arrows indicate mCherry puncta that appeared as single large puncta or as clusters, respectively, and do no co-localize with GFP, possibly corresponding to peroxisomes that have undergone pexophagy. Scale bars = 20 \( \mu \text{m} \).
the protein and potentially allowing recruitment of pexophagy machinery.

In plants expressing GMT, I detected puncta with only mCherry fluorescence in root tips and hypocotyls (Fig. 5.6B, Fig. 5.7B, C, D), indicating that GMT may be useful for monitoring pexophagy in Arabidopsis. I did not observe a large change in GMT localization from 4- to 7-day-old seedlings, but I only noticed consistent presence of puncta with only mCherry fluorescence in hypocotyls and root tips, indicating that pexophagy may be higher in these tissues than in cotyledons. Moreover, I observed these mCherry-only puncta in the center of the cell, which is consistent with vacuolar localization of peroxisomes that have undergone pexophagy. I also observed diffuse red fluorescence in some hypocotyl cells (Fig. 5.7B, D), which might reflect peroxisomes that have been degraded in the vacuole. As the puncta with only mCherry fluorescence are slightly larger than the average puncta with both GFP and mCherry fluorescence (Fig. 5.6B, Fig. 5.7B, C, D), it is tempting to speculate that the puncta with only mCherry fluorescence are similar in nature to the large puncta observed in lon2 mutants.

Experiments to validate the identity of these puncta are needed. Crossing a GMT-expressing line to atg mutants could determine the necessity of autophagy for these puncta. Treatment of a GMT-expressing line with concanamyin A, which inhibits vacuolar acidification, could also be informative; if these puncta correspond to peroxisomal membranes and not some autofluorescence, then I expect that GFP will co-localize with these larger puncta following treatment with concanamycin A. It would also be interesting to see if the degradation product observed in the MT lines (Fig. 5.4A) was autophagy dependent, which could provide a gel-based marker for pexophagy.

I crossed pUBQ10:GMT T3-6B3 to lon2-2, atg7-4, and the lon2-2 atg7-4 double mutant but failed to isolate homozygous lon2 and atg7 mutants that carried the pUBQ10:GMT T3-6B3 transgene. I germinated the F2 seeds of the various crosses on media containing Basta to select for plants that were expressing the transgene. For the crosses to the lon2 mutants, I added IBA along with Basta to the media to select for plants that were IBA resistant (and therefore likely homozygous for the lon2 mutation). Of the more than 100 F2 seeds of the crosses to the lon2 mutants, none were IBA resistant although approximately two-thirds of the seedlings were resistant to Basta. I genotyped the F2 plants and identified plants that were heterozygous for the lon2 mutations and contained the transgene. These results indicate that the crosses between the transgene-containing plant and the lon2 mutants were successful but that the transgene is linked
to LON2, which is on the bottom arm of Chromosome 5. ATG7 is tightly linked to LON2 on the bottom of chromosome 5, and I was only able to recover transgene-expressing F2 plants that were heterozygous for atg7-4, which is consistent with the hypothesis that the transgene is on the bottom of Chromosome 5. I thereafter crossed an independent transgenic line (pUBQ10:GMT T3-5G3) to lon2-2, lon2-8, atg7-4, and the lon2-2 atg7-4 double mutant. I also crossed pUBQ10:GMT T3-6B3 to atg18a-3 as ATG18a is on Chromosome 3. The F1 plants have been moved to soil, and progeny of the desired genotypes are expected provided the transgene in the T3-5G3 does not also reside on Chromosome 5.

GMT and MT will be useful for future studies of pexophagy and peroxisome biology. I expect that lon2-2 expressing GMT will have an increased ratio of mCherry-only puncta relative to wild type and that atg and lon2-2 atg will have no mCherry-only puncta. Crossing GMT into various lon2 alleles (Fig. 4.1A) or lines expressing a lon2 variant (Fig. 4.5) could provide insight into how LON2 regulates pexophagy by monitoring the prevalence of pexophagy in these different backgrounds. Crossing GMT into various pex mutants could be informative of the role of each peroxin in pexophagy. Many of the pex mutants in the Bartel lab collection have import defects and display cytosolic fluorescence of GFP with a peroxisomal matrix targeting signal, and crossing MT-expressing lines into various pex mutants will enable visualization of changes in peroxisome morphology that may occur in these mutants.
Chapter 6: Conclusions and future prospects

Parts of this chapter are published (Young and Bartel, 2016).

Peroxisome quality control is an intricate process that is poorly understood, but our understanding of how plant cells orchestrate this process continues to grow. My studies have uncovered new alleles of autophagy-related genes in Arabidopsis (Chapter 3), expanded knowledge of the functions of LON2 (Chapter 4), and developed lon2 protein variants (Chapter 4) and a tandem-fluorescent membrane reporter (Chapter 5) that will enable further investigation into how plants maintain peroxisome quality control.

6.1. Screening for additional autophagy and pexophagy components

Arabidopsis provides an attractive platform to discover molecular components of autophagy because plants lacking autophagy are viable in standard growth conditions (Doelling et al., 2002). Several ATG genes have yet to be characterized in Arabidopsis due to a lack of mutants defective in these genes, and many details of selective autophagy, including pexophagy, are unknown. Screening for lon2 suppressors is a highly tractable system to discover autophagy components in an unbiased and facile manner, and my efforts yielded 26 novel alleles in six different ATG genes (Table 3.1). Two of these six ATG genes (ATG3 and ATG16) do not have insertional alleles available in public repositories. The atg alleles that I identified will be useful for future investigations of autophagy in Arabidopsis. Additional screening for lon2 suppressors has the potential to identify not only known homologs of ATG genes but also currently unknown plant-specific and pexophagy-specific genes. For example, the whole-genome sequencing of lon2-2 suppressor L70 did not reveal mutations in any genes known to be important for autophagy, and identification of the causal lesion in L70 may reveal a plant-specific autophagy component or a gene required specifically for pexophagy in Arabidopsis. Screening for chemicals that suppress lon2-2 IBA resistance (discussed in Chapter 4) affords an opportunity to identify chemicals that can inhibit autophagy in plants with greater specificity and efficacy than currently used autophagy inhibitors.

Alternative forward-genetic screens may uncover pexophagy-specific components in Arabidopsis. Although nbr1 mutants do not suppress lon2-2, this result does not exclude the
possibility that NBR1 might act redundantly in pexophagy. Thus, a screen for *lon2 nbr1* suppressors might uncover a pexophagy receptor acting redundantly with NBR1 in Arabidopsis. A microscopy-based screen for mutants with altered GFP-mCherry-PEX26TM hypocotyl fluorescence (Fig. 5.7) might uncover genes that either negatively or positively regulate pexophagy. Moreover, as a microscopy-based screen for altered GFP-mCherry-PEX26TM fluorescence would not rely on peroxisomal metabolism (IBA sensitivity), mutants in gene important for both peroxisome function and pexophagy, such as *pex* mutants, might emerge from this screen.

### 6.2. The signals for pexophagy remain to be identified

The signals that mark peroxisomes for pexophagy are not identified in plants. Although nonfunctional catalase accumulates in *atg2* mutants, *atg2 cat2 cat3* triple mutants, which lack detectable catalase, still exhibit the clustered peroxisome phenotype associated with *atg2* mutants (Shibata et al., 2013). This result indicates that aggregated catalase is not a necessary signal for pexophagy. The observation that expressing *lon2* with a functional AAA domain and a nonfunctional protease domain prevents the excessive pexophagy of *lon2* mutants (Goto-Yamada et al., 2014) suggests that the chaperone function of LON2 suppresses pexophagy. This suppression supports a hypothesis that misfolded or aggregated matrix protein(s) may signal for pexophagy. Studies of the *lon2* protein variants that I generated (Chapter 4) will likely provide insight not only into the functions of LON2 but also into key players involved in peroxisome quality control. Examination of the suite of *lon2* phenotypes in *lon2*-8 mutants expressing the *lon2* variants will provide insight into which domains of LON2 are important for various functions. I expect that experiments using a substrate-trapping *lon2* variant will identify LON2 substrates that include signals for pexophagy as I hypothesize that accumulation of a currently unidentified LON2 substrate acts as the signal that stimulates pexophagy in *lon2* mutants. Subsequent reverse-genetic analyses of candidate substrates might provide further insight into the roles of these genes in peroxisome quality control. Such a signal would presumably need to traverse the peroxisome membrane to be recognized by a cytosolic pexophagy receptor (Fig. 1.2D). Moreover, the pexophagy receptor may require the PEX4 ubiquitin conjugating enzyme and the PEX2/PEX10/PEX12 ubiquitin-protein ligases (Fig. 1.1C) to ubiquitinate the misfolded or aggregated protein prior to recognition.
Peroxins could also serve directly as pexophagy receptor targets. Although PEX3, PEX5, and PEX14 have been implicated in pexophagy in yeast and mammals (Motley et al., 2012; Deosaran et al., 2013; Farré et al., 2013; Yamashita et al., 2014; Burnett et al., 2015; Jiang et al., 2015; Zhang et al., 2015; Zientara-Rytter et al., 2017), similar roles for these peroxins have not been demonstrated in plants.

The GFP-mCherry-PEX26TM tandem-fluorescent peroxisome membrane reporter that I developed (Chapter 5) will be a useful tool for future studies studying pexophagy in Arabidopsis. This reporter can be crossed into different peroxisomal mutants, allowing observation of the relative rates of pexophagic flux in these mutants. Based on the observation of pexophagic flux in different mutants, inferences can be drawn on the importance of each gene for regulating pexophagy. Mutants of genes that prevent pexophagy would be expected to have a higher ratio of mCherry:GFP-mCherry puncta, indicating a higher rate of pexophagic flux, and mutants of genes that promote pexophagy would be expected to have a lower ratio of mCherry:GFP-mCherry puncta, indicating a lower rate of pexophagic flux. Thus, the tandem-fluorescent reporter could enable identification of genes that are positive or negative regulators of pexophagy in plants.

6.3. Other proteins important for pexophagy and selective autophagy receptors in Arabidopsis have yet to be characterized

Atg37 is important for pexophagy in yeast (Nazarko et al., 2014; Zientara-Rytter et al., 2017) but has not been identified in plants. Atg37 is an acyl-CoA binding protein that is thought to help recruit and regulate phosphorylation of the pexophagy receptor Atg30 (Nazarko et al., 2014; Zientara-Rytter et al., 2017). The mammalian homolog of Atg37, known as acyl-CoA–binding domain containing protein 5 (ACBD5), is also implicated in pexophagy (Nazarko et al., 2014), and mutants of this gene have defects in fatty acid β-oxidation (Yagita et al., 2017).

Intriguingly, Oryza sativa Acyl-CoA Binding Protein 6 (ACBP6) localizes to peroxisomes, and expression of Oryza sativa ACBP6 in Arabidopsis pxa1 mutants complements pxa1 β-oxidation defects (Meng et al., 2014), suggesting that Oryza sativa ACBP6 can bypass the need for Arabidopsis PXA1. Arabidopsis contains six different acyl-CoA binding proteins (reviewed in Lung and Chye, 2016), but PXA1 is not included in these six ACBPs. Although none of these ACBPs is reported to localize to peroxisomes, two ACBPs localize to membranes (reviewed in Lung and Chye, 2016). Perhaps PXA1 or one of the membrane-localized ACBPs in Arabidopsis
plays a critical role in pexophagy.

Although my results suggest that NBR1 is not a pexophagy receptor for lon2 peroxisomes in Arabidopsis, NBR1 acts as a selective autophagy receptor in Arabidopsis (Svenning et al., 2011; Zhou et al., 2013; Zhou et al., 2014). Further studies of NBR1 might uncover a role for NBR1 in specific instances of pexophagy. For example, pex1 and pex6 mutants display heightened pexophagy in Arabidopsis (Rinaldi et al., 2017; Gonzalez et al., 2018), yeast (Nuttall et al., 2014), and humans (Law et al., 2017) perhaps as a result of accumulation of ubiquitinated proteins in the peroxisomal membrane, and NBR1 may be critical for the detrimental pexophagy observed in pex1 and pex6 mutants. A better understanding of NBR1 functions could also provide insight into how plant cells maintain quality control of organelles besides peroxisomes. Identifying NBR1 interactors might elucidate the role of NBR1 in other types of selective autophagy, such as mitophagy (selective autophagy of mitochondria), in Arabidopsis, and identifying NBR1 interactors might also indicate that NBR1 plays a role in specific instances of pexophagy if peroxisome-related proteins are discovered to interact with NBR1. Crossing Arabidopsis expressing 35S:YFP-NBR1 to an atg7 mutant would prevent degradation of NBR1 and any interacting proteins and enable pull down of YFP-NBR1 using an antibody to YFP, which could be followed by mass spectrometry to identify proteins bound to YFP-NBR1. Crossing Arabidopsis expressing 35S:YFP-NBR1 to the tandem-fluorescent construct pUBQ10:GFP-mCherry-PEX26TM may uncover a role for NBR1 in pexophagy; YFP-NBR1 may co-localize with peroxisomes undergoing pexophagy, and overexpressing NBR1 may induce pexophagy as monitored by the ratio of GFP-mCherry puncta to mCherry puncta.

Pull down of GFP-ATG8a and GFP-ATG11 may also reveal pexophagy components in Arabidopsis. Selective autophagy receptors often bind to ATG8a (reviewed in Rogov et al., 2014), so the pexophagy receptor might be identified as an ATG8a-interacting protein. Moreover, PEX14 directly binds to LC3-II (mammalian homolog of lipidated ATG8) in mammals (Jiang et al., 2015), so other proteins critical for pexophagy may be identified as ATG8a-interacting proteins. Indeed, Arabidopsis ATG8f binds to PEX6 and PEX10 (Xie et al., 2016). In P. pastoris, Atg11 binds to Atg8 and to the pexophagy receptor Atg30 (Farré et al., 2013), so it will be interesting to determine if ATG11 plays a similar role in Arabidopsis pexophagy. Crossing plants expressing either GFP-ATG8a or GFP-ATG11 to atg18a-3 would allow ATG8 lipidation but prevent isolation membrane expansion and closure, preventing
autophagic degradation of GFP-ATG8a or GFP-ATG11 and any interacting proteins. Pulling down GFP-ATG8a or GFP-ATG11 could be performed using an antibody recognizing GFP, and interacting proteins could be identified using mass spectrometry. Immunoblotting could be used to test for potential interacting proteins for which we have antibodies (e.g., PEX5, PEX6, PEX10, and PEX14), and interactions could be confirmed using yeast-two-hybrid studies or reciprocal co-immunoprecipitation experiments where antibodies exist. Proteins interacting with GFP-ATG8a or GFP-ATG11 might be involved in pexophagy or in other selective autophagy pathways.

6.4. Concluding Remarks

The core autophagy machinery is conserved across kingdoms. Plants, fungi, and metazoans all utilize autophagy to maintain intracellular quality, recycle nutrients, and respond to stress. The oxidative nature of peroxisomes is also conserved and necessitates a mechanism to turn over these vital organelles, and pexophagy performs this function not only in fungi and animals but also in plants.

Considering the conservation of autophagic pathways, the similarities between pexophagy in plants and other organisms likely outweigh the differences. Although pexophagy receptors are often unique to particular organisms, all examined systems are unified in utilizing a receptor protein to link condemned peroxisomes to the autophagy machinery. It will be exciting to discover the molecular components executing pexophagy in plants as well as the triggers that promote pexophagy in response to environmental and developmental changes. Moreover, it will be interesting to apply the knowledge gained from the study of plant peroxisomes, such as the role of the LON2 peroxisomal protease in preventing pexophagy, to other organisms.
References


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