Plant autophagy—more than a starvation response
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Autophagy is a conserved mechanism for the degradation of cellular contents in order to recycle nutrients or break down damaged or toxic material. This occurs by the uptake of cytoplasmic constituents into the vacuole, where they are degraded by vacuolar hydrolases. In plants, autophagy has been known for some time to be important for nutrient remobilization during sugar and nitrogen starvation and leaf senescence, but recent research has uncovered additional crucial roles for plant autophagy. These roles include the degradation of oxidized proteins during oxidative stress, disposal of protein aggregates, and possibly even removal of damaged proteins and organelles during normal growth conditions as a housekeeping function. A surprising regulatory function for autophagy in programmed cell death during the hypersensitive response to pathogen infection has also been identified.

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Introduction
Autophagy (‘self-eating’) is a universal mechanism in eukaryotic cells for digestion of cell contents to recycle needed nutrients, degrade damaged or toxic components, or to reclaim cellular materials as a precursor to cell death. In plants, two major autophagic pathways have been described, microautophagy and macroautophagy [1]. In microautophagy, material is engulfed directly by the vacuole via invagination of the tonoplast, followed by pinching off of the membrane to release a vesicle containing the cytoplasmic constituents inside the vacuole lumen. Microautophagy-like processes have been observed during the deposition of storage proteins in developing wheat seeds [2,3] and release of rubber particles into the vacuole [4], though these processes do not result in immediate degradation of the material deposited into the vacuole. Microautophagy also occurs in some species during seed germination for degradation of starch granules and storage protein in vacuoles [5,6]. Macroautophagy, by contrast, is initiated in the cytoplasm with the formation of cup-shaped membranes of unknown source that enclose the material to be degraded. The membranes elongate and eventually fuse together to produce a double-membrane autophagosome containing cytoplasmic material. The outer autophagosomal membrane fuses with the tonoplast, releasing an autophagic body consisting of the inner membrane and contents into the vacuole. Vacular acid hydrolases then degrade the autophagic body, and the degradation products are presumably transported back to the cytosol [1]. The functions of macroautophagy in suspension cultures and whole plants in response to sucrose and nitrogen starvation and during senescence have been well described for a number of plant species (for example, see references [7–10]). The recent development of markers to rapidly and easily detect the occurrence of autophagy in plant cells by the specific labeling of autophagosomes has facilitated the study of autophagy. These markers include a fusion of green fluorescent protein (GFP) with the autophagosome-localized protein ATG8 [11–13] and the acidotrophic fluorescent dyes monodansylcadaverine (MDC [11]) and LysoTracker Red [14]. In this review, I will summarize advances over the past two years in our understanding of macroautophagy, hereafter simply termed autophagy, and in particular focus on additional physiological roles for autophagy in plants that are now coming to light.

Pathways for autophagy in plants
Examination of the morphological characteristics of autophagy in different plant species has led to some discrepancies regarding the precise pathway and site of degradation of cytoplasmic material (Figure 1). For example, when the cysteine protease inhibitor E-64 was used to block autophagosomal degradation in Arabidopsis and barley, cytoplasmic inclusions accumulated as autophagic bodies within the central vacuole, whereas in tobacco they accumulated in smaller organelles outside the central vacuole that are termed autolysosomes [15]. Extensive analyses of the stages of autophagosome formation and delivery to the vacuole has suggested that in plants, two distinct pathways exist in which autophagosomes can either fuse directly with the vacuole or can first be engulfed by a smaller lysosome-like or endosome-like organelle, which begins the degradation of the contents, before eventual fusion with the vacuole [16,17]. The species and conditions under investigation may determine which pathway is predominant at any given time.

Arabidopsis proteins required for autophagy
On the basis of sequence similarity to yeast proteins required for autophagy [18], a number of Arabidopsis...
proteins can be predicted to function in autophagy, primarily in autophagosome formation [1,19]. While a large amount of information is now available on the yeast proteins, in many cases their precise function in autophagy is still unknown. Most of the autophagy proteins can be classified into several major complexes or processes, including protein kinases involved in the initiation or regulation of autophagosome formation, a phosphatidylinositol 3-kinase complex, two ubiquitin-like conjugation systems and the ATG9 complex, which may function in membrane recruitment to the site of autophagosome formation (Table 1).

The best studied proteins involved in plant autophagy are those of the ubiquitin-like conjugation systems [19]. The first involves the formation of a covalently linked conjugate of ATG5 and ATG12. The reaction is reminiscent of the attachment of ubiquitin to proteins to tag them for degradation, though the ATG5-ATG12 conjugate is stable and seems to be the functional form of the two proteins. The conjugation enzymes show similarity to those of the ubiquitin pathway, consisting of an E1-type enzyme ATG7 and an E2-like ATG10. While ATG12 does not have any sequence homology with ubiquitin, the recent crystal structure of Arabidopsis ATG12 reveals that the protein has a canonical ubiquitin fold [20]. The second system is more unusual in that the ATG8 protein is conjugated to a lipid, phosphatidylethanolamine (PE), rather than a protein. The same ATG7 E1 enzyme performs this reaction, along with an E2, ATG3. A processing enzyme that cleaves the C-terminus of ATG8 is also required. Both of these systems are required for autophagosome formation in Arabidopsis [12,13,20], and knockout mutants in several of the corresponding genes have been identified and show starvation sensitivity and early senescence phenotypes as expected for disruption of autophagy [8,12,13,20].

Components of the ATG9 complex have also been studied in Arabidopsis using genetic approaches. Atg9 is an integral membrane protein that may be required for the delivery of membrane to the forming autophagosome [18], and disruption of the Arabidopsis ATG9 gene results in typical autophagy phenotypes [9]. In yeast, Atg9 interacts with Atg2, and Atg18 is required for the correct localization of both of these proteins. Disruption of ATG2 or ATG18 in Arabidopsis also prevents autophagosome formation [15,21], suggesting conserved functions for the proteins in plants.

Not all proteins required for autophagy are specific for that pathway. For example, in yeast, Atg6 is required for biosynthetic trafficking to the vacuole [22] in addition to being a component of the phosphatidylinositol 3-kinase complex required for autophagy [23], and the SNARE Vti1 is probably required for all vesicle transport pathways terminating at the vacuole [24]. Several homologs of Vti1 with partially overlapping functions are present in Arabidopsis [25], and VTI12 is thought to be the homolog required for autophagy [26]. ATG6 also appears to be a multi-functional protein in plants, and a knockout mutant in the Arabidopsis ATG6 gene is defective in pollen tube germination [27,28], probably because of a vacuolar trafficking defect. In tobacco, an ATG6 homolog is involved in the regulation of programmed cell death, and this function is related to a role in autophagy (see below [29]).
**Autophagy during oxidative stress**

In addition to its role in nutrient recycling during starvation, autophagy may be a more general response to a variety of abiotic stress conditions. Exposure of Arabidopsis seedlings to oxidative stress, either by direct addition of H₂O₂ or by addition of methyl viologen (MV) to generate reactive oxygen via the photosynthetic electron transport chain, led to a rapid and strong induction of autophagy [30**](Figure 2). RNAi-*AtATG18a* transgenic plants, which are defective in autophagosome formation because of decreased expression of an *ATG18* homolog, are hypersensitive to oxidative stress, indicating a physiological role for autophagy in response to this stress. One potential reason for the hypersensitivity was revealed by the analysis of oxidized proteins in wild-type and RNAi-*AtATG18a* plants under oxidative stress conditions. Oxidized proteins accumulated in RNAi-*AtATG18a* plants, and their rate of degradation was reduced compared with that of wild-type plants, suggesting that autophagy is required for the degradation of damaged cellular components. In situ localization of oxidized proteins provided further evidence for this, as when vacuolar degradation was blocked using ConA, oxidized proteins accumulated in the vacuole in wild-type plants, whereas they remained in the cytoplasm in RNAi-*AtATG18a* plants (Figure 3). Together, these data indicate that, during oxidative stress, oxidized proteins and potentially other cell components are transferred to the vacuole by autophagy for degradation [30**]. Whether autophagy also plays a role in clearing damaged proteins during other abiotic stresses remains to be seen.

**Autophagy during programmed cell death**

While under abiotic stress conditions autophagy is generally considered to be a response to assist in cell survival, autophagy is also well known to contribute to programmed cell death (PCD) by degradation of cellular contents before death [31]. Classical apoptosis as seen in animal cells is unlikely to occur in most plant systems, as the presence of the cell wall would preclude engulfment of cellular remains by surrounding cells. By contrast, many instances of PCD in plants show typical morphological features of autophagic cell death, including an increase in vacuole and cell size, uptake of organelles into the vacuole followed by organelle degradation, and eventual lysis of the vacuole resulting in cell death. These characteristics have been described by electron microscopic observations in such diverse systems as soybean and foxglove nectaries upon termination of nectar production [32,33] and death of suspensor cells during embryogenesis in gymnosperms [34].

Vacuolar proteases with caspase-like activity are regulators of PCD in response to a variety of pathogens [35–37]. A role for a caspase-type protease activity in autophagic PCD has been indicated recently in barley seed development [38]. While the protein responsible for the activity has yet to be identified, the protease activity correlated with PCD and was inhibited by caspase-specific inhibitors. *In situ* labeling with fluorescent substrate demonstrated the localization of the protease to vesicles that were also labeled with the autophagosome marker MDC [38], providing an intriguing connection between the protease and autophagy activity. Whether this protease is a regulator of autophagy, is involved in degradation of cellular components during autophagic cell death, or has some other role is not yet clear.

A role for autophagy in the response to pathogen infection is clear in animals [39], but recent research indicates an
unexpected function in the innate immune response in tobacco. Upon infection by tobacco mosaic virus (TMV), infected cells undergo PCD in a process termed the hypersensitive response, as a means of limiting pathogen spread. Using a high-throughput virus-induced gene silencing (VIGS) screen, Liu et al. discovered that a tobacco homolog of the autophagy protein Atg6 (also called Beclin1) is involved in TMV-induced PCD [29**]. The tobacco BECLIN1 gene appears to function in autophagy, as it was able to complement the yeast atg6 mutant phenotype, and silenced plants showed an accelerated senescence phenotype, typical of autophagy mutants. Whereas the cell death lesions during the TMV-induced hypersensitive response are normally restricted to the infected cells, VIGS of BECLIN1 (or other autophagy gene homologs) caused expanded lesions that extended beyond the infection site and were even present in uninfected upper leaves. These results suggested that BECLIN1 is required for the restriction of PCD to the TMV infection site.

Arabidopsis Atg6 homologs have autophagy-independent functions [27,28], possibly in trafficking to the vacuole as in yeast. The effect of BECLIN1 on cell death, however, does appear to involve the autophagy pathway. In non-silenced infected plants, autolysosomes accumulated at the infection site and were also seen in non-infected leaves, suggesting that autophagy may be induced systematically during TMV infection [29**]. Silencing of BECLIN1 prevented this induction of autophagy. The same phenotype was observed upon infection with bacterial and fungal pathogens, indicating that autophagy is involved in the hypersensitive response in general, rather than a specific response to TMV. The mechanism by which autophagy prevents the spread of cell death is still unknown. One possibility is that autophagy degrades a death induction signal, preventing it from spreading to uninfected cells. Alternatively, autophagy may degrade components damaged by reactive oxygen species in adjacent cells, thereby preventing death.

A role for autophagy in the absence of stress conditions
It has been speculated that autophagy is involved in the disposal and degradation of abnormal or damaged organelles and proteins, even under normal growth conditions without the imposition of environmental stresses. This has come into question because of the lack of an obvious growth phenotype of Arabidopsis autophagy mutants throughout most of their life cycle [8,9,12,13,21]. With the use of the protease inhibitor E-64d, it has now been demonstrated that autophagy does occur under nutrient-rich conditions, at least in certain cell types [14,15]. When Arabidopsis or barley root tips were incubated in the presence of nutrients and E-64d, cytoplasmic inclusions were seen to accumulate inside vacuoles in the root meristem and in the elongation zone, suggesting that autophagy was active in these cells. These barley autophagic vacuoles were unusual in containing the tonoplast marker α-TIP, and not δ-TIP or γ-TIP, suggesting functional specialization of the vacuoles [14].

The mechanism of uptake of cytoplasm under normal conditions was addressed by incubation of root tips in the presence of the autophagy inhibitor 3-methyladenine (3-MA) [40]. 3-MA partially inhibited the accumulation of cytoplasm inside the vacuole [15*]. With the use of the protease inhibitor E-64d, it has now been demonstrated that autophagy does occur under nutrient-rich conditions, at least in certain cell types [14,15*]. When Arabidopsis or barley root tips were incubated in the presence of nutrients and E-64d, cytoplasmic inclusions were seen to accumulate inside vacuoles in the root meristem and in the elongation zone, suggesting that autophagy was active in these cells. These barley autophagic vacuoles were unusual in containing the tonoplast marker α-TIP, and not δ-TIP or γ-TIP, suggesting functional specialization of the vacuoles [14].
constitutive autophagy in root tips as for stress-induced autophagy.

A potential role for constitutive autophagy in degrading damaged proteins was suggested by the examination of oxidized proteins and lipids in Arabidopsis plants defective in autophagy. In plants, reactive oxygen species are commonly generated via the photosynthetic electron transport chain, and some oxidative damage can occur even under standard growth conditions. In RNAi-atATG18a plants, which are defective in autophagy, an increased level of oxidized proteins and lipids was observed when compared with wild-type plants [42], indicating that autophagy may participate in the degradation of these oxidized molecules. In addition, autophagy-defective plants contain increased levels of reactive oxygen species and enhanced expression of reactive oxygen scavenging enzymes [30*,42], suggesting that they are constitutively under a degree of oxidative stress. One possible explanation is that these plants are unable to dispose of damaged organelles, which therefore continue to produce reactive oxygen species.

Morphologically, the formation of vacuoles in meristematic cells appears to involve an autophagy process, in which vesicles and tubules fuse around portions of cytoplasm, eventually forming an autophagosome-like structure in which the cytoplasmic contents are degraded [1,43]. Surprisingly, Arabidopsis knockout mutants defective in autophagy do not show developmental phenotypes, other than early senescence, even though vacuole formation is essential for embryo development in Arabidopsis [44]. These disparate observations have been at least partially reconciled in recent work by Yano et al. [45*], who used a mini-protoplast system to study vacuole biogenesis. Mini-protoplasts lacking large central vacuoles were prepared from tobacco BY-2 protoplasts and incubated for one to two days, during which time large vacuoles reformed. In the presence of cysteine protease inhibitors the newly formed vacuoles contained cytoplasmic material, suggesting an autophagy process is also operational in vacuole formation in these cells. The autophagy inhibitors 3-MA and wortmannin, which block starvation-induced autophagy in BY-2 cells [40] and also constitutive autophagy in Arabidopsis root tips [15*], had no effect on vacuole formation in the mini-protoplasts [45*]. Together, these results suggest that autophagy is involved in vacuole biogenesis, but that the mechanism of autophagy is distinct from the conserved pathway of stress-induced or constitutive autophagy involving the ATG genes.

Evidence for selective autophagy

Autophagy is in general considered to be non-selective, with autophagosomes enclosing random portions of cytoplasm for delivery to the vacuole. However, some intriguing recent data have led to the suggestion that in some cases the autophagic machinery may selectively transport specific types of cargo at a faster rate than the bulk cytoplasm. A fusion between cytochrome b5 and red fluorescent protein (Cytb5-RFP) was observed to form punctate aggregates in the cytoplasm when expressed in tobacco cells under normal growth conditions [16**]. By contrast, during nitrogen limitation, the fusion protein was found in the central vacuole and processed to a lower molecular weight form. Cytb5-RFP fluorescence in the cytoplasm co-localized with the autophagosome marker YFP-ATG8, suggesting that Cytb5-RFP was taken up into autophagosomes, and transport to the vacuole was blocked by 3-MA. Together these data indicate that the transport occurs via autophagy.

Two lines of evidence suggest that there may be a degree of selectivity in the transport of Cytb5-RFP. First, the degradation rate of Cytb5-RFP was compared with that of other proteins, and Cytb5-RFP was found to be processed by vacuolar enzymes faster than other proteins. Second, the degree of co-localization with YFP-ATG8 was higher for Cytb5-RFP than for mitochondria, suggesting Cytb5-RFP is incorporated into autophagosomes at a higher frequency. The authors conclude that autophagy may be selective for protein aggregates, like those formed by Cytb5-RFP [16**]. The mechanism behind the selectivity is not known and could be due to direct recognition of aggregated protein or, more generally, the shape or size of the aggregates formed. While some questions remain regarding the results reported (for example, the stability of the Cytb5-RFP fusion in the vacuole is not known nor is its rate of synthesis, both of which could affect the interpretation of the results), this study provides the first indication that autophagy may be capable of selectively removing certain proteins or structures in plant cells.

Conclusions

In addition to known functions in nutrient recycling, autophagy in plants has now been shown to play diverse roles in responses to abiotic and biotic stresses, programmed cell death and vacuole formation. Many important questions remain: Are there additional examples of selective autophagy in plant cells, and how are substrates selected for vacuolar delivery? What is the relationship between autophagy and vacuole formation, and which proteins are required for the formation of vacuoles during development? What determines the switch from autophagy as a stress survival response to autophagy as a programmed cell death pathway? How is autophagy induced and regulated in these different circumstances? Many exciting advances can be anticipated in the future as these and other questions are addressed and our understanding of the roles of autophagy in plants is enhanced.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

● of special interest
●● of outstanding interest


7. Aubert S, Gout E, Bligny R, Marty Mazars D, Barrieu F, Olsen LJ, Yoshimoto K: Starvation-induced autophagy inhibitor 3-methyladenine and Arabidopsis knockout mutants barley root tips even in the presence of nutrients. The use of the autophagy inhibitor 3-methyladenine and Arabidopsis knockout mutants in autophagy genes demonstrated that this constitutive autophagy occurs by the same pathway as starvation-induced autophagy, although its precise function remains to be shown.


This work demonstrates a requirement for autophagy in the degradation of oxidized proteins during oxidative stress in Arabidopsis. Autophagy was induced by reactive oxygen species, and disruption of autophagy led to the accumulation of oxidized proteins. In situ detection of oxidized proteins showed that they are taken up into the vacuole for degradation by autophagy.


The authors show that the formation of vacuoles in evacuolated protoplasts prepared from tobacco cells involves an autophagy-like process, but that this autophagy is resistant to classical autophagy inhibitors, suggesting that it occurs by a distinct mechanism.