Degradation of Oxidized Proteins by Autophagy during Oxidative Stress in Arabidopsis[1][W][OA]

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Upon encountering oxidative stress, proteins are oxidized extensively by highly reactive and toxic reactive oxidative species, and these damaged, oxidized proteins need to be degraded rapidly and effectively. There are two major proteolytic systems for bulk degradation in eukaryotes, the proteasome and vacuolar autophagy. In mammalian cells, the 20S proteasome and a specific type of vacuolar autophagy, chaperone-mediated autophagy, are involved in the degradation of oxidized proteins in mild oxidative stress. However, little is known about how cells remove oxidized proteins when under severe oxidative stress. Using two macroautophagy markers, monodansylcadaverine and green fluorescent protein-AtATG8e, we here show that application of hydrogen peroxide or the reactive oxidative species inducer methyl viologen can induce macroautophagy in Arabidopsis (*Arabidopsis thaliana*) plants. Macroautophagy-defective RNAi-*AtATG18a* transgenic plants are more sensitive to methyl viologen treatment than wild-type plants and accumulate a higher level of oxidized proteins due to a lower degradation rate. In the presence of a vacuolar H^+-ATPase inhibitor, concanamycin A, oxidized proteins were detected in the vacuole of wild-type root cells but not RNAi-*AtATG18a* root cells. Together, our results indicate that autophagy is involved in degrading oxidized proteins under oxidative stress conditions in Arabidopsis.

Reactive oxidative species (ROS), the partially reduced or activated derivatives of oxygen, are highly reactive and toxic and can lead to cell death by causing damage to proteins, lipids, carbohydrates, and DNA (Mittler et al., 2004). There are many potential sources of ROS in plants. Under normal physiological conditions, ROS are continuously produced in mitochondria, chloroplasts, and peroxisomes as the byproducts of aerobic metabolic processes such as respiration and photosynthesis. ROS production can be enhanced by many abiotic stresses, such as drought stress, salt stress, heat shock, low temperature, nutrient deprivation, and high light (Malan et al., 1990; Prasad et al., 1994; Tsugane et al., 1999; Desikan et al., 2001; Mittler, 2002). ROS can increase during some developmental stages (e.g. senescence; Woo et al., 2004). In addition, some biotic stresses, such as pathogen infection and wounding, also trigger a ROS burst produced by NADPH oxidase-, amine oxidase-, or cell wall-bound peroxidase-dependent pathways. These ROS then act as signaling molecules to activate stress response and defense pathways (Chen and Schopfer, 1999; Orozco-Cardenas and Ryan, 1999; Torres et al., 2002).

Due to the dual roles of ROS in toxicity and as signal molecules, plant cells have developed sophisticated strategies to regulate their intracellular ROS concentration and to detoxify excess ROS. These strategies can be divided into two categories: avoidance mechanisms and scavenging mechanisms (for review, see Mittler, 2002). Avoidance strategies, such as leaf movement and curling and D1 protein degradation leading to PSII photoinactivation in high light conditions, enable plants to avoid excess ROS production (Okada et al., 1996; Mullineaux and Karpinski, 2002). Scavenging strategies use numerous scavenging enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase, and catalase (CAT) or use nonenzymatic antioxidants such as ascorbate and glutathione to detoxify the excess ROS (Noctor and Foyer, 1998; Apel and Hirt, 2004). Most attention so far has been paid to these scavenging strategies. Studies of knockout, overexpression, and antisense plants of many scavenging enzymes have strongly indicated that these scavenging enzymes are involved in plant growth, development, and different biotic and abiotic stress responses by regulating intracellular ROS concentration. Reduced expression of CAT1 in tobacco (*Nicotiana tabacum*), for example, causes hydrogen peroxide (H_2O_2) accumulation and induces cell death in palisade parenchyma cells in high light conditions (Dat et al., 2003). Overexpression of thylakoidal

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APX in Arabidopsis (*Arabidopsis thaliana*) increases resistance to methyl viologen (MV)-induced photooxidative stress (Murgia et al., 2004).

Once the generation of ROS exceeds these avoidance and scavenging mechanisms, oxidative stress occurs and causes damage to cellular components such as proteins. How plant cells degrade these damaged, oxidized proteins is unclear. The proteasome-dependent proteolytic system, because of its selectivity, has been considered to be a possible candidate for removing oxidized proteins. In mammalian cells, the 20S proteasome, the proteolytic core of the 26S proteasome complex, is involved in the degradation of oxidatively modified proteins, and this degradation is ATP and ubiquitin independent (Grune et al., 1995; Ullrich et al., 1999; Dunlop et al., 2002; Shringarpure et al., 2003). In plants, the 20S proteasome has been isolated from maize (*Zea mays*) roots and can be activated by mild oxidative conditions, such as sugar starvation (Basset et al., 2002), although a direct role in degradation of oxidized proteins was not shown.

Another major proteolytic system that could potentially degrade oxidized proteins is autophagy. Autophagy is a process in which cytoplasmic components are taken up into the vacuole or lysosome for degradation. There are three major forms of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA; Cuervo, 2004). In microautophagy, the cytosolic components are engulfed directly by the vacuolar membrane. In macroautophagy, cytoplasmic components are surrounded by a double membrane structure to form an autophagosome. The outer membrane of the autophagosome then fuses with the vacuole and the inner membrane and its contents are degraded by vacuolar hydrolases. In some conditions, whole organelles, such as mitochondria and peroxisomes, and parts of organelles, such as regions of Golgi and endoplasmic reticulum, are removed by the macroautophagy pathway (Cuervo, 2004). Macro- and microautophagy are generally thought to have no selectivity. In contrast, CMA can selectively degrade cytosolic proteins by using a cytosolic chaperone, cyt-hsc70, to recognize substrate proteins containing the signal motif KFERQ (Massey et al., 2004). Recently, CMA has been found to participate in removing oxidized proteins in rat liver and cultured mouse fibroblasts under oxidative stress (Kiffin et al., 2004). Whether macroautophagy or microautophagy contributes to the degradation of oxidized proteins is unclear. Although some yeast (*Saccharomyces cerevisiae*) autophagy mutants, such as *atg3*, are more sensitive to oxidative stress compared to wild type (Thorpe et al., 2004), there is still no direct evidence to support a role for macroautophagy in the removal of oxidized proteins during oxidative stress.

In plants, only macroautophagy (referred to hereafter as autophagy) and microautophagy have been identified and it is unclear whether CMA is present in plants (Thompson and Vierstra, 2005). By sequence comparison, autophagy genes (ATGs) are well conserved among yeasts, animals, and plants, indicating that the basic molecular mechanism of autophagy is also likely to be conserved in higher eukaryotes (Mizushima et al., 1998; Liang et al., 1999; Doelling et al., 2002; Hanaoka et al., 2002). Phenotypic analysis of Arabidopsis knockout and RNAi silenced lines of several homologs of yeast ATGs have suggested that autophagy is involved in nutrient deprivation responses and in regulating the senescence process (Doelling et al., 2002; Hanaoka et al., 2002; Xiong et al., 2005). Furthermore, two useful autophagosome markers, monodansylcadaverine (MDC) and green fluorescent protein (GFP)-AtATG8, have been developed for use in Arabidopsis (Yoshimoto et al., 2004; Contento et al., 2005; Thompson et al., 2005). MDC is a fluorescent dye that specifically stains autophagosomes, whereas AtATG8 proteins are localized to autophagosomes after being modified with the lipid phosphatidylethanolamine by a ubiquitination-like reaction. These two markers now allow us to monitor the autophagic process in Arabidopsis. Here, using these two autophagosome markers, we show that autophagy is induced after treatment with H_2O_2 or MV. Autophagy-defective RNAi-*AtATG18a* transgenic plants are more sensitive to MV treatment and accumulate a higher level of oxidized proteins compared to wild type. Our data suggest that autophagy is involved in degrading oxidized proteins during oxidative stress in Arabidopsis.

RESULTS

Autophagy Is Induced by MV or H_2O_2 Treatment

Although autophagy has been suggested to be involved in resistance to oxidative stress in yeast (Thorpe et al., 2004), the function of autophagy during oxidative stress and whether plant cells also use autophagy as a defense against oxidative stress is unclear. In a previous study, we characterized one marker for autophagy in Arabidopsis, the fluorescent dye MDC, which specifically stains autophagosomes (Contento et al., 2005). To investigate whether oxidative stress can induce autophagy in Arabidopsis, we first examined the response of wild-type plants to two well-known oxidative stress inducers, H_2O_2 and MV. Whereas H_2O_2 can diffuse across membranes and cause damage directly to cellular components, MV acts by accepting electrons from PSI in chloroplasts and then reacting with oxygen to produce superoxide. Seven-day-old wild-type seedlings grown on nutrient Murashige and Skoog (MS) solid medium were transferred to the same medium plus 10 μM MV for 2 d or MS solid medium plus 10 mM H_2O_2 for 6 h, then stained with MDC. Numerous motile MDC-stained autophagosomes were detected in root cells after MV or H_2O_2 treatment, observed by fluorescence microscopy (Fig. 1, B and C). These MDC-stained autophagosomes were absent from seedlings grown on control medium (Fig. 1A). These observations imply that autophagy can be induced by oxidative stress.

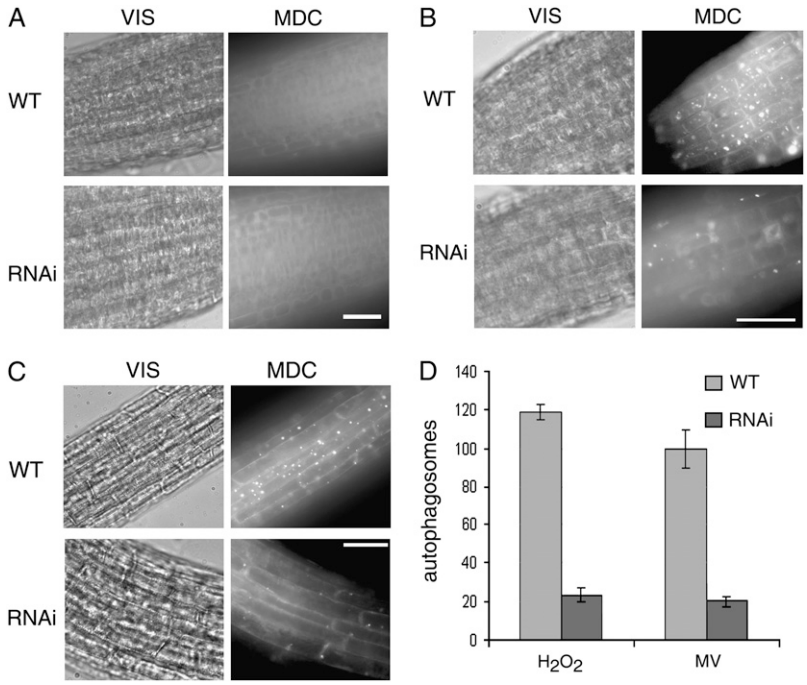

Figure 1. MDC staining of root tips. Seven-day-old wild-type (WT) and RNAi-*AtATG18a* seedlings were transferred to nutrient control solid MS medium for 2 d (A), to nutrient solid MS medium containing 10 μM MV for 2 d (B), or to nutrient solid MS medium containing 10 mM H_2O_2 for 6 h (C), followed by staining with MDC, and observed by fluorescence microscopy. Scale bar = 50 μm. D, Number of autophagosomes per root section were counted after MV or H_2O_2 treatment as above and the average number determined for 10 seedlings per genotype and treatment. Error bars indicate SE.

AtATG8 proteins have been shown to associate with autophagosomes, and GFP-AtATG8 fusion proteins have been used as markers of autophagosomes in Arabidopsis (Yoshimoto et al., 2004; Contento et al., 2005; Thompson et al., 2005). To further confirm the MDC staining results, we introduced a transgene expressing *GFP-AtATG8e* into wild-type ecotype Columbia plants. When grown on nutrient MS solid medium, GFP-AtATG8e was dispersed throughout the cytoplasm, and punctate autophagosome-like structures were not visible (Contento et al., 2005; Supplemental Fig. S1). When these transgenic *GFP-AtATG8e* plants were transferred for 2 d to solid MS medium with 10 μM MV, numerous punctate structures were observed in root cells, similar to the MDC-stained punctate structures (Supplemental Fig. S1), indicating the presence of autophagosomes. Together, these results strongly suggest that autophagy is induced by oxidative stress.

RNAi-*AtATG18a* Plants Are More Sensitive to MV Treatment

The AtATG18a protein is required for autophagosome formation in Arabidopsis, and its transcript is up-regulated both during Suc and nitrogen starvation and during senescence. RNAi-*AtATG18a* transgenic lines with reduced *AtATG18a* expression show hypersensitivity to Suc and nitrogen starvation and are unable to produce autophagosomes under these conditions (Xiong et al., 2005). To analyze the expression pattern of *AtATG18a* under oxidative stress conditions, total RNA was isolated from wild-type and RNAi-*AtATG18a* seedlings after incubation with 10 μM MV for up to 72 h, or in the absence of MV as a control, and reverse transcription (RT)-PCR was performed using primers specific for *AtATG18a*. As shown in Figure 2, the transcript level of *AtATG18a* increased after MV treatment in wild-type seedlings. Although this expression increase was also seen in RNAi-*AtATG18a* seedlings, the level of *AtATG18a* transcript is much lower than in wild-type seedlings (Fig. 2). To determine whether autophagosome formation is disrupted in RNAi-*AtATG18a* root cells under oxidative stress, 7-d-old RNAi seedlings were transferred to solid MS medium plus 10 μM MV for 2 d or plus 10 mM H_2O_2 for 6 h, then stained with MDC (Fig. 1, B and C). Compared to wild-type seedlings, fewer MDC-stained autophagosomes were detected in the root tips of these RNAi seedlings (Fig. 1D). Both H_2O_2 and MV treatment caused an increase in ROS in wild-type and RNAi seedlings, as measured by Amplex Red measurement of H_2O_2 concentration, indicating that the lack of autophagosome production in the RNAi plants is not due to decreased production of ROS in these plants (Supplemental Fig. S2). Overall, RNAi-*AtATG18a* seedlings had higher ROS levels than wild type, possibly due to an increase in damaged organelles caused by the autophagy defect in these lines. These data suggest that AtATG18a is not only required in starvation- and senescence-induced autophagy, but also in oxidative stress-triggered autophagy.

To determine whether autophagy is required for the response to oxidative stress conditions, growth of the RNAi-*AtATG18a* lines was compared with wild-type plants under oxidative stress. Seven-day-old seedlings grown on nutrient MS solid medium (Fig. 3A) were transferred to medium with 10 μM MV (Fig. 3B). MV strongly inhibited growth of both wild-type and RNAi plants (compare with Xiong et al., 2005). However,

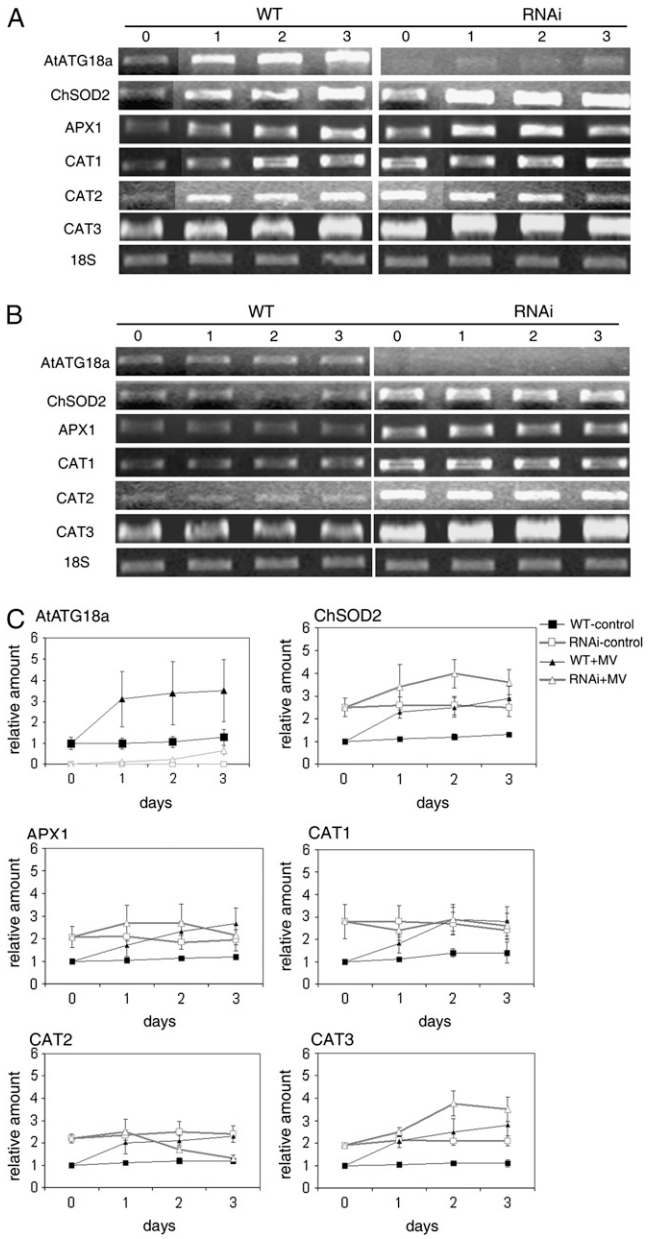

Figure 2. Gene expression analysis upon MV treatment. Seven-day-old wild-type (WT) and RNAi-*AtATG18a* seedlings were transferred to either solid MS medium containing 10 μM MV (A) or to control medium (B) for the indicated times. Total RNA was isolated and RT-PCR was performed using gene-specific primers. 18S RNA was used as a loading control. ChSOD2, Chloroplast SOD. C, The relative amounts of RT-PCR product were quantified by densitometry from three independent repeats, with the wild-type control value set as 1. Error bars indicate SE.

after 15 d, RNAi-*AtATG18a* plants were already chlorotic, whereas wild-type seedlings were still green (Fig. 3). In contrast, silencing of an unrelated gene by RNAi had no effect on response to oxidative stress (data not shown). These data imply that autophagy is necessary for plants to survive oxidative stress conditions.

To assess whether the RNAi-*AtATG18a* lines are likely to be under increased oxidative stress, the expression patterns of several ROS scavenging enzymes were compared between wild-type and RNAi-*AtATG18a* seedlings. RNA was extracted from seedlings after incubation in the presence (Fig. 2A) or absence (Fig. 2B) of MV for 72 h followed by RT-PCR analysis and quantification by densitometry (Fig. 2C). As expected, in wild-type seedlings, transcript abundance increased in the presence of MV for each gene, whereas it remained constant in control samples. In RNAi-*AtATG18a* seedlings, the transcript level was higher in each case than in wild-type plants, even in the absence of MV, possibly suggesting that the RNAi seedlings are under increased or constitutive stress compared with wild type. In the presence of MV, the mRNA levels for CAT3, APX, and SOD increased still further, demonstrating that the RNAi seedlings are still able to respond to increased ROS concentrations. The CAT1 mRNA level remained constant in the RNAi lines, and CAT2 transcript level decreased at later time points, possibly reflecting different roles or differential regulation of CAT isoforms in response to oxidative stress.

Autophagy Is Responsible for Degradation of Oxidized Proteins during Oxidative Stress

One result of oxidative stress is increased protein oxidation. To better understand the role of autophagy in oxidative stress, the level of oxidized proteins was compared between wild-type and RNAi-*AtATG18a* seedlings after MV treatment. Derivatization with 2,4-dinitrophenylhydrazine (DNPH) was used, as this assay is well established for use in plant systems (e.g. Romero Puertas et al., 2002; Davletova et al., 2005; Guo and Crawford, 2005). Seven-day-old seedlings were transferred to medium containing 10 μM MV for up to 12 h, followed by isolation of total proteins. After derivatization of the carbonyl group of oxidized proteins with DNPH, immunoblotting was performed

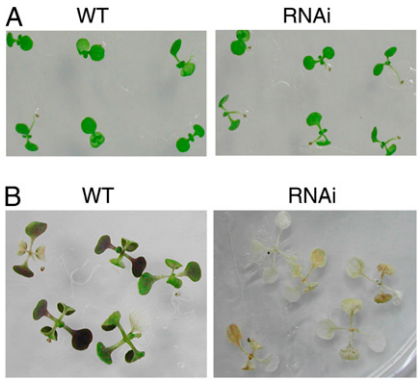

Figure 3. Phenotype of RNAi-*AtATG18a* plants under oxidative stress conditions. Seven-day-old seedlings of wild-type (WT) and RNAi-*AtATG18a* plants (A) were transferred to MS medium containing 10 μM MV for 15 d (B).

using 2,4-dinitrophenol (DNP) antibodies to detect oxidized proteins. The signal on the blot was quantified by densitometry of the entire lane of the gel. As shown in Figure 4, RNAi plants accumulated approximately 3.5-fold more oxidized proteins compared with wild-type seedlings after MV treatment (compare wild type + MV with RNAi + MV in Fig. 4, A and C; $P = 0.0085$). Similar results were obtained by quantifying either the entire lane or individual prominent bands (data not shown), with no evidence for selective increases in specific proteins. No signal was seen in parallel control experiments in which the DNP derivatization step was omitted (data not shown). Considering that RNAi-*AtATG18a* seedlings are defective in autophagy, these data indicate that inhibition of autophagy leads to increased accumulation of oxidized proteins during oxidative stress.

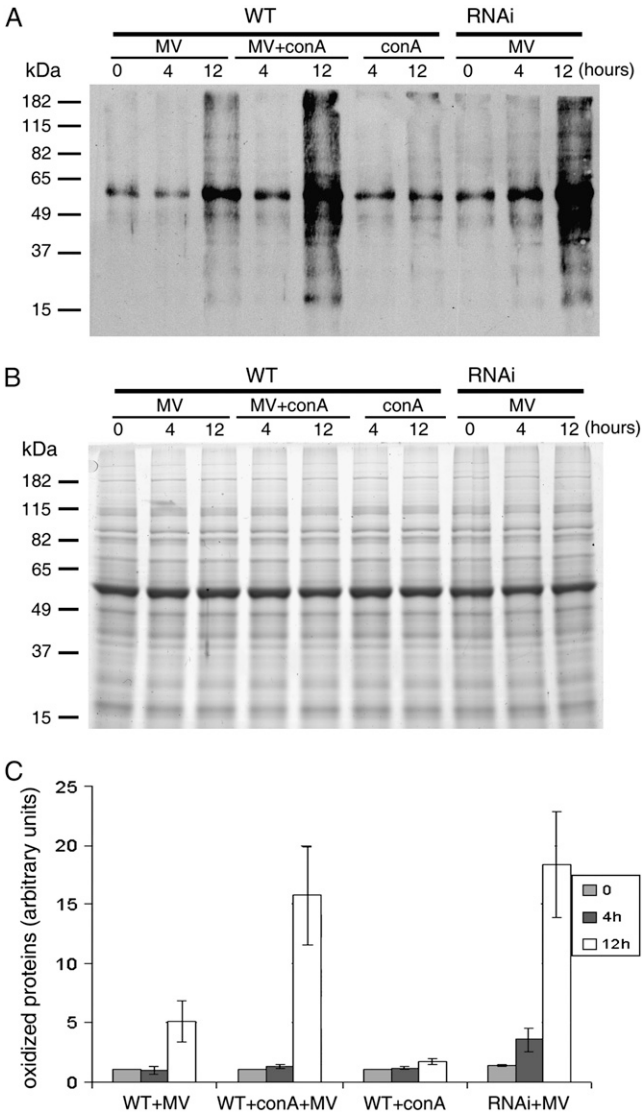

Figure 4. Protein oxidation analysis. A, Seven-day-old seedlings of wild-type (WT) and RNAi-*AtATG18a* plants were transferred to solid medium containing 10 μM MV for the indicated hours. For concanamycin A (conA) treatment, wild-type seedlings were transferred to solid medium containing 1 μM concanamycin A for the indicated times. Total proteins were isolated and derivatized by DNP followed by immunoblotting using DNP antibody. Molecular size markers are indicated at the left. B, Coomassie Blue-stained gel of samples from part A to demonstrate equal loading. Molecular size markers are indicated at the left. C, DNP signals from part A were quantified by densitometry from three independent repeats with the wild-type control value set as 1; error bars indicate SE.

We considered two possibilities to explain the increased accumulation of oxidized proteins in RNAi-*AtATG18a* seedlings during MV treatment: either an increased production of oxidized proteins or a decrease in their degradation. To distinguish between these possibilities, we analyzed the effect of concanamycin A on the level of oxidized proteins after MV treatment. Concanamycin A is a vacuolar H^+-ATPase inhibitor, which inhibits vacuolar enzyme activities by increasing the internal vacuolar pH and therefore prevents vacuolar protein degradation (Drose et al., 1993). When treated with concanamycin A, autophagosomes accumulate in the vacuole in Arabidopsis roots, as they can no longer be degraded (Yoshimoto et al., 2004). Seven-day-old wild-type seedlings were transferred to medium containing 10 μM MV or 1 μM concanamycin A, or both 10 μM MV and 1 μM concanamycin A for up to 12 h, followed by protein isolation and immunoblotting using DNP antibodies. MV and concanamycin A treatment caused a significant increase in accumulation of oxidized proteins compared to MV treatment alone in wild-type seedlings (approximately 3-fold; Fig. 4, A and C), and this accumulation level is similar to that in RNAi-*AtATG18a* lines when treated with MV. Treatment of RNAi-*AtATG18a* seedlings with MV and concanamycin A had no effect compared to MV alone (Supplemental Fig. S3), indicating that the RNAi and concanamycin A most likely inhibit the same protein degradation pathway. These data indicate that the increased accumulation of oxidized proteins in RNAi-*AtATG18a* seedlings during MV treatment is most likely caused by the decrease in their degradation, not by the increased production of oxidized proteins.

To further confirm that the increased accumulation of oxidized proteins in RNAi seedlings is caused primarily by reduced degradation of these proteins by autophagy, the rate of degradation of oxidized proteins was compared between wild-type and RNAi plants. Seedlings were treated with 10 μM MV for 2 d, by which time oxidized proteins had accumulated to high levels in both wild-type and RNAi-*AtATG18a* seedlings. The seedlings were then transferred back to solid medium lacking MV, and the degradation rate of oxidized proteins in wild-type and RNAi plants was compared. Initially, the amount of oxidized protein detected remained constant or increased slightly (Fig. 5), most likely due to the continued presence of MV in the seedlings even after transfer back to control medium. As shown in Figure 5, 3 or 4 d after transfer back to control medium, the amount of oxidized proteins in

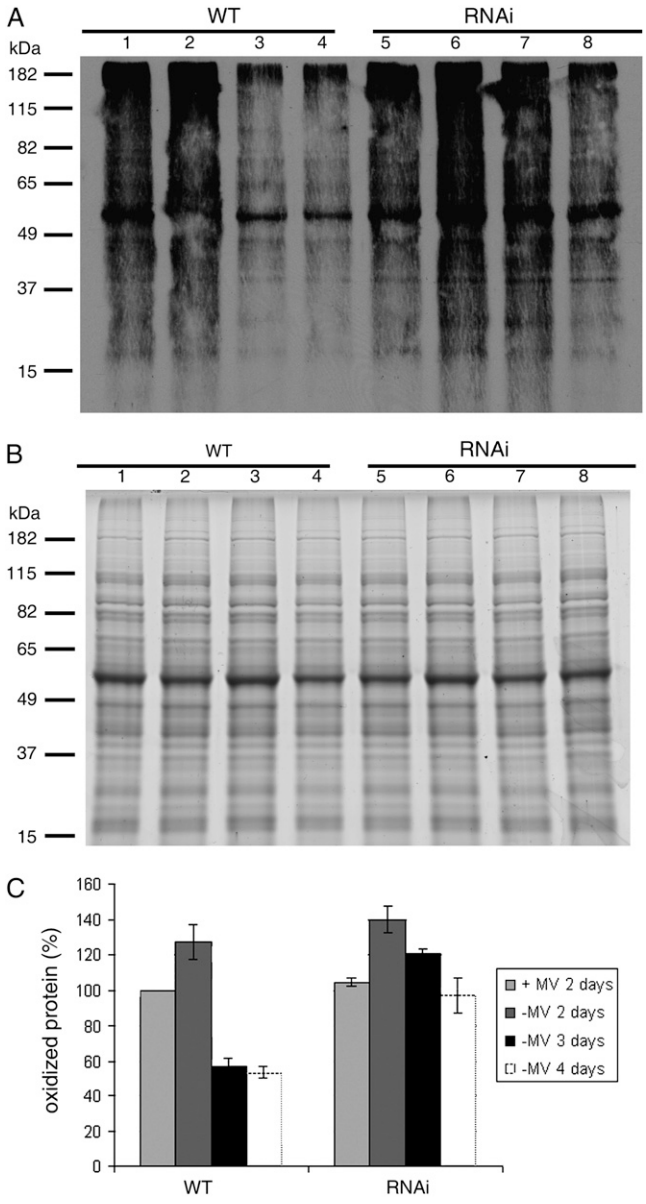

Figure 5. Degradation of oxidized proteins. A, Seven-day-old seedlings of wild-type (WT) and RNAi-*AtATG18a* plants were transferred to solid medium containing 10 μM MV for 2 d (lanes 1 and 5) then transferred back to normal solid MS medium and incubated for 2 d (lanes 2 and 6), 3 d (lanes 3 and 7), or 4 d (lanes 4 and 8). Total proteins were isolated and derivatized by DNP followed by immunoblotting using DNP antibody. Molecular size markers are indicated at the left. B, Coomassie Blue-stained gel of samples from part A to demonstrate equal loading. Molecular size markers are indicated at the left. C, DNP signals from part A were quantified from three independent repeats, with the zero-time wild-type signal (lane 1) set as 100%; error bars indicate SE.

wild-type seedlings had decreased to a low level. In contrast, RNAi-*AtATG18a* plants retained a significantly higher level of oxidized proteins at this time point after transfer (2-fold increase; $P = 0.002$ for the 3-d time point). Together, these data suggest that oxidized proteins can be transported to the vacuole for degradation by the autophagy pathway. In this way, cells can eliminate oxidized proteins to increase their survival under severe oxidative stress.

Oxidized Proteins Are Delivered to the Vacuole for Degradation

To further confirm that during oxidative stress oxidized proteins can be sent to the vacuole for degradation by the autophagy pathway, the cellular localization of oxidized proteins caused by MV treatment was compared between wild-type and RNAi-*AtATG18a* root cells. Three-day-old wild-type and RNAi-*AtATG18a* seedlings were transferred to medium containing 1 μM concanamycin A to inhibit degradation in the vacuole, or both 1 μM concanamycin A and 10 μM MV for 12 h. The oxidized proteins were then derivatized in situ with DNP and detected using DNP antibody followed by immunofluorescence confocal microscopy. In the expanded root cells shown, the vacuole occupies most of the cell volume, and the cytoplasm is present as a thin layer adjacent to the plasma membrane. After treatment with 1 μM concanamycin A and 10 μM MV for 12 h, a strong DNP signal was detected inside the vacuole of wild-type root cells (Fig. 6). In contrast, this DNP signal was absent from the vacuole of RNAi-*AtATG18a* root cells and instead seen in the cytoplasm (see Supplemental Fig. S4 for three-dimensional [3D] image). This provides direct evidence that autophagy is responsible for transporting oxidized proteins to the vacuole for degradation under oxidative stress conditions.

DISCUSSION

As a nonspecific protein degradation pathway, autophagy is activated in response to some environmental stresses, such as nutrient deficiency, and during certain stages of development (e.g. senescence; Levine and Klionsky, 2004). In this study, several lines of evidence support the hypothesis that autophagy can also be induced for removal of oxidized proteins during

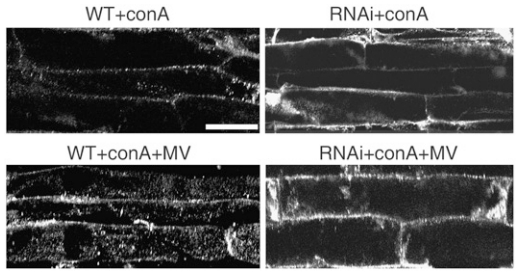

Figure 6. Immunofluorescence analysis of the cellular localization of oxidized proteins. Three-day-old wild-type (WT) and RNAi-*AtATG18a* seedlings grown vertically on solid medium were transferred to solid medium containing 1 μM concanamycin A (conA) or both 1 μM concanamycin A and 10 μM MV (MV + conA) and incubated for 12 h. The oxidized proteins were derivatized with DNP and detected using DNP antibodies, followed by confocal microscopy. Scale bar = 20 μm.

oxidative stress in Arabidopsis. Using two autophagosome markers, MDC and GFP-AtATG8e, numerous autophagosomes were detected in wild-type root cells after 2 d of MV treatment, but only a few autophagosomes were observed in autophagy-defective RNAi-*AtATG18a* transgenic plants (Fig. 1B), implying that autophagy is induced by oxidative stress. RNAi-*AtATG18a* seedlings were more sensitive to oxidative stress compared to wild type (Fig. 3), implying that autophagy is necessary for plants to survive such oxidative stress conditions. After treatment with MV for 12 h, more oxidized proteins accumulated in RNAi-*AtATG18a* seedlings than in wild-type seedlings (Fig. 4), and in RNAi-*AtATG18a* seedlings, the degradation rate of oxidized proteins was also slower than in wild type (Fig. 5), implying that autophagy is responsible for degrading these oxidized proteins. In wild-type seedlings, more oxidized proteins accumulated after treatment with MV and concanamycin A together compared to treatment with MV only (Fig. 4), suggesting a role for the vacuole in degradation of oxidized proteins. Under oxidative stress, when treated with concanamycin A, oxidized proteins can be detected in the vacuole of wild-type root cells but not RNAi-*AtATG18a* root cells (Fig. 6; Supplemental Fig. S4). These data provide direct evidence that oxidized proteins are sent to the vacuole for degradation by autophagy. In addition, two different sources of ROS (direct addition of H_2O_2 and generation in the chloroplast by MV) both cause autophagy, suggesting that it is general oxidative stress that induces autophagy rather than localized ROS production in a specific organelle. Based on these observations, we hypothesize that one of the physiological roles of autophagy during oxidative stress is to remove oxidized proteins.

In mammalian cells, the 20S proteasome and CMA pathway were found to degrade oxidized proteins selectively; the 20S proteasome recognizes hydrophobic regions on the surface of target proteins and is ATP and ubiquitin independent (Dunlop et al., 2002), whereas the CMA pathway uses a pentapeptide KFERQ in proteins as a signal for translocation into the lumen of the lysosome after being unfolded (Kiffin et al., 2004). Furthermore, the efficiency of the 20S proteasome and CMA pathway in removal of oxidized proteins changes with the duration and degree of oxidative damage. Mild oxidative damage causes exposure of hydrophobic regions and partial protein unfolding, facilitating the degradation of the oxidized proteins by the 20S proteasome and CMA pathways. In contrast, under severe oxidative stress, oxidized proteins easily aggregate and cross-link together, decreasing the efficiency of the 20S proteasome and CMA pathways (Dunlop et al., 2002; Kiffin et al., 2004). How mammalian cells remove oxidized proteins rapidly and effectively under severe oxidative stress is unknown, and whether macroautophagy contributes to their degradation is unclear. In plants, a CMA pathway has not been demonstrated. The 20S proteasome has been isolated from maize roots and is activated by carbonylation modification in mild oxidative conditions, such as carbon starvation, although inactivated by strong oxidative treatments such as a high concentration of H_2O_2. However, no direct evidence was provided as to whether this enhanced proteasome activity is related to degradation of oxidized proteins (Basset et al., 2002). These data imply that the 20S proteasome plays an important role in removal of oxidized proteins under mild oxidative stress but not under severe oxidative stress. However, plants frequently suffer severe oxidative stress, because they cannot escape from unfavorable environments (Millar et al., 2001). Considering that a large population of proteins is oxidized and that these proteins aggregate and are cross-linked during severe oxidative stress, it is plausible that plant cells use autophagy to remove these damaged proteins rather than the selective 20S proteasome pathway. Because autophagy is nonselective and does not require a targeting signal that could be inaccessible in aggregated and cross-linked proteins, it could effectively transfer oxidized proteins to the vacuole for degradation.

Previously, we showed that AtATG18a is required for autophagosome formation, and autophagosomes were absent in RNAi-*AtATG18a* seedlings under nutrient deprivation and senescence conditions (Xiong et al., 2005). When treated with MV, however, a few MDC-stained autophagosomes can still be observed in RNAi-*AtATG18a* roots (Fig. 1B). This difference may be due to the nature of RNAi technology; there is still a very low level of mRNA remaining in the RNAi seedlings, and under MV treatment, the *AtATG18a* transcript level increased somewhat in the RNAi plants (Fig. 2). This suggests the expression of *AtATG18a* in these RNAi plants is not completely silenced. Alternatively it could be due to the differing extent of autophagy induced by nutrient deprivation, senescence, and oxidative stress. More autophagosomes were seen after MV or H_2O_2 treatment than during nutrient deprivation conditions (Y. Xiong and D.C. Bassham, unpublished data), implying that the extent of autophagy during oxidative stress is higher than under nutrient deprivation and senescence conditions. This may allow the detection of a few autophagosomes in RNAi plants during oxidative stress but not in nutrient deprivation and senescence conditions.

In yeast, most of the *ATG* are single copy. In contrast, some Arabidopsis homologs of yeast ATGs exist as a small gene family. For example, there are eight homologs for ATG18 (Xiong et al., 2005) and nine homologs for ATG8 (Hanaoka et al., 2002). One hypothesis is that autophagy induced by different stresses or different developmental stages may require different AtATG homologs. However, the same ATG18 homolog, AtATG18a, is required for nutrient depletion-, senescence-, and oxidative stress-induced autophagy. This implies that the molecular mechanism of autophagy is the same in senescence, nutrient deprivation, and oxidative stress. We have isolated T-DNA knockout mutants for four additional ATG18 homologs: *AtATG18c*, *AtATG18e*, *AtATG18f*, and *AtATG18g*. Autophagosome formation

detected by MDC staining was not affected in these knockout mutants during senescence, nutrient deprivation, and oxidative stress conditions (Xiong et al., 2005; Y. Xiong and D.C. Bassham, unpublished data), implying that these genes are not required for already known autophagy functions. Of course, care must be taken in drawing this conclusion, as there may be some redundant functions between these AtATG18 homologs, and knockout of one gene may not be enough to observe a clear phenotype. Generating double or triple mutants will help to address this question.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) plants were grown on nutrient solid MS medium as described previously (Xiong et al., 2005) under long-day conditions (16 h light) at 22°C. Arabidopsis seeds were surface sterilized in 33% (v/v) bleach and 0.1% (v/v) Triton X-100 solution for 20 min followed by cold treatment for at least 2 d. For MV, H_2O_2, or concanamycin A treatment, 7-d-old seedlings grown on nutrient solid MS medium were transferred to the same medium containing 10 μM MV, 10 mM H_2O_2, or 1 μM concanamycin A and incubated for the indicated times.

Staining of Seedlings with MDC and Microscopy

Seedlings were stained with MDC as described (Contento et al., 2005) and observed using fluorescence microscopy using a 4′,6-diamino-phenylindole-specific filter. For counting of autophagosomes, pictures were taken of equivalent regions of each root for multiple seedlings after MDC staining. The number of autophagosomes visible in each image was counted and the average determined for 10 seedlings per genotype and per treatment.

H_2O_2 Measurement

H_2O_2 was measured in extracts from Arabidopsis seedlings according to Rao et al. (2000). Leaves were frozen, ground to a powder, and stored at −80°C. Leaf powder (50–100 mg) was extracted with 0.1 mL of 0.2 M $HClO_4$, incubated on ice for 5 min, and then centrifuged at 14,000*g* for 10 min at 4°C. The supernatant was neutralized with 0.2 M NH_4OH, pH 9.5, and was centrifuged again at 3,000*g*. The extracts were passed through 1- × 1-cm columns of AG 1 × −8 resin (Bio-Rad) and were eluted with 1 mL water.

The quantification of H_2O_2 in the extracts was performed using the Amplex Red Hydrogen Peroxide-Peroxidase Assay kit (Invitrogen-Molecular Probes) following the manufacturer's directions. Fluorescence was measured with a Synergy HT fluorescence plate reader (Bio-Tek) using excitation at 530 nm and fluorescence detection at 590 nm. The concentration of H_2O_2 in each sample was calculated using a standard curve.

RT-PCR Analysis of Expression of *AtATG18a* and ROS-Scavenging Enzymes

Total RNA was extracted and RT-PCR was performed as described previously (Xiong et al., 2005). Gene-specific primers used for PCR are as follows: *AtATG18a* (At3g62770), 5′-TCGCGTCGACTCCTTCAAATCATTCT-TCCATG-3′ and 5′-TCGCTCTAGATTAGAAAACTGAAGGCGGTTT-3′; *CAT1* (At1g20630), 5′-CACATGTTTTCATTTCTCTTGATGAT-3′ and 5′-AGCAGA-CAATAGGAGTTGTAGGG-3′; *CAT2* (At4g35090), 5′-AACATGTTTTCATTT-CTCTTGATGAT-3′ and 5′-AACAGACAGCAGGCGGAGTTGGA-3′; *CAT3* (At1g20620), 5′-GTCGACATGGATCCTTACAAGTATCGTCCTTCAAGC-3′ and 5′-GCGGCCGCCTAGATGCTTGGCCTCACGTTCAGACGGCT-3′; *ChlSOD2* (At2g28190), 5′-ATTTCCTCCAAACGTCAAACAT-3′ and 5′- CATCGGCATTGG-CATTTATGT-3′; and *APX1* (At1g07890), 5′-ACGTTCTCATTCATGACTCTATAT-3′ and 5′-GGAAATCAGCAAAAGAGATGGTA-3′.

RT-PCR signals were quantified by densitometry of individual bands using Quantity One software (Bio-Rad) using the volume analysis function, and the relative signals were calculated, normalized to a wild-type control value of one for each gene individually. The results shown are an average of three independent experiments.

Generation of GFP-AtATG8e Transgenic Plants

A DNA fragment encoding GFP-AtATG8e was obtained by digestion of plasmid pJ4GFP-AtATG8e (Contento et al., 2005) with restriction enzymes *Sal*I and *Sac*I and ligated into the plant T-DNA binary vector pCAMBIA1300 (Cambia GPO). This construct was introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation (Merereau et al., 1990). pCAMBIA1300: GFP-AtATG8e was introduced into Arabidopsis ecotype Columbia plants by Agrobacterium-mediated transformation using the floral dipping method (Clough and Bent, 1998). Transgenic plants were screened using hygromycin resistance and expression confirmed by immunoblotting using GFP antibody (Invitrogen). Homozygous T2 transformant seeds were used for further studies.

Oxidized Protein Analysis

For oxidized protein analysis, total proteins were isolated from seedlings after treatment with MV for the indicated times by grinding in cold extraction buffer (0.1 M Tris-HCl, pH 7.5, 0.3 M Suc, 1 mM EDTA, 0.1 mM phenylmethylsulphonylfluoride, and 1% [v/v] β-mercaptoethanol) and centrifugation at 1,000*g* for 10 min. Oxidized proteins were detected using an OxyBlot protein oxidation detection kit (Chemicon International) according to the manufacturer's instructions. DNP signals were quantified by densitometry using Quantity One software (Bio-Rad) using the volume analysis function, and the relative signals were calculated. In Figure 4, signals were normalized to a wild-type 0-h control value of one. Signals were determined both for the entire lane of gel and for individual prominent protein bands, with essentially equivalent results for both methods. The results shown are an average of three independent experiments for quantification of the entire gel lane. In Figure 5, signals were normalized to the wild type + MV signal, which was set as 100%.

Unpaired *t* tests were conducted on the protein oxidation data to determine statistical significance. Significant difference was defined by treatment comparisons with observed *P* values of less than 0.05 ($P < 0.05$), allowing for a 95% confidence interval.

Immunolocalization and Confocal Microscopy

Three-day-old Arabidopsis seedlings grown on MS solid medium were transferred to the same medium containing 1 μM concanamycin A or 1 μM concanamycin A plus 10 μM MV for 12 to 16 h. Immunofluorescence staining of treated Arabidopsis seedlings was performed according to Muller et al. (1998) with the following modifications: Seedlings were fixed with Carnoy's solution (6 parts absolute ethanol, 1 part glacial acetic acid, 3 parts chloroform) for 15 min followed by successive washes with 50% (v/v) ethanol, 25% (v/v) ethanol and 50 mM PIPES, 5 mM EGTA, and 5 mM $MgSO_4$ (MTSB), then mounted on slides. Prior to addition of block buffer (3% [w/v] bovine serum albumin/MTSB), permeabilized seedlings were treated with 40 μL 1 × DNPH (Chemicon International) for 30 min and neutralized with 40 μL neutralization solution (OxyBlot protein oxidation kit, Chemicon International). Immunolabeling was done using anti-DNP (OxyBlot protein oxidation kit, Chemicon International; 1:100) for 15 to 18 h at 4°C followed by Alexa Fluor 594-conjugated goat anti-rabbit secondary antibodies (Molecular Probes; 1:250) for 1 h at room temperature. Samples were washed five times with MTSB after each antibody. Images were collected using a Leica TCS/NT confocal microscope (Leica Microsystems), and 3D reconstruction was performed using MetaMorph (Molecular Devices Corporation) and Adobe Photoshop.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Subcellular localization of GFP-AtATG8e.

Supplemental Figure S2. H_2O_2 measurement in seedlings.

Supplemental Figure S3. Concanamycin A has no effect on levels of oxidized proteins in RNAi-*AtATG18a* plants.

Supplemental Figure S4. 3D image of immunofluorescence analysis of the cellular localization of oxidized proteins.

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