

Reactive Oxygen Species and Autophagy in Plants and Algae¹

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Reactive oxygen species (ROS) and autophagy have been historically associated with cell death. However, more recent evidence indicates that both ROS and autophagy play important roles in signaling and cellular adaptation to stress. As a catabolic process, autophagy allows eukaryotic cells to recycle intracellular components including entire organelles during development or under stress conditions such as nutrient limitation. Degradation and recycling of macromolecules via autophagy provides a source of building blocks (amino acids, lipids, and sugars) that allow temporal adaptation of cells to adverse conditions. In addition to recycling, autophagy is required for the degradation of damaged or toxic material that can be generated as a result of ROS accumulation during oxidative stress. The mitochondrial electron-transport chain and the peroxisomes are primary sources of ROS production in most eukaryotes. The plant cell contains an additional organelle, the chloroplast, with an intense electron flow that leads to high rates of ROS production. Studies in plants and algae have demonstrated that autophagy is structurally and functionally conserved in photosynthetic organisms and plays an important role in the cellular response and adaptation to different stress conditions that involve the generation of ROS, such as oxidative and drought stresses, pathogen infection, or photooxidative damage. These findings suggested a strong link between autophagy and ROS in photosynthetic eukaryotes. Here, we review recent studies in plants and algae describing redox control of autophagy and discuss the conserved regulatory proteins that may transmit redox signals to the autophagic machinery.

AUTOPHAGY: GENERAL FEATURES

During autophagy (also known as macroautophagy), cytoplasmic components are nonselectively enclosed within a double-membrane vesicle known as the autophagosome and delivered to the vacuole/lysosome for degradation of toxic components and recycling of needed nutrients (He and Klionsky, 2009; Mizushima et al., 2011; Liu and Bassham, 2012). This degradative process is widely conserved through evolution, and accordingly, autophagy-related (ATG) genes have been found in all eukaryotes. Originally identified in the budding yeast *Saccharomyces cerevisiae* (Tsukada and Ohsumi, 1993), homologs to ATG genes have been described from lower eukaryotes like fungi and algae to plants and metazoans (Meijer et al., 2007; Díaz-Troya et al., 2008b). Some ATG proteins have a structural function in autophagy, while others are important for the regulation of the process. For instance, the ATG5-ATG12-ATG16 protein complex and the ATG8-phosphatidylethanolamine (PE) conjugate are essential for autophagosome formation and completion, while ATG8-PE also mediates fusion of the autophagosome to the vacuole membrane (Mizushima et al., 2011). The ATG4 Cys protease processes nascent ATG8 at the C terminus to facilitate its covalent binding to PE, but it is also able to cleave PE from ATG8 for its recycling, playing a crucial role in autophagy regulation (Kirisako et al., 2000; see below). Another important regulatory protein in the autophagic process is the ATG1 kinase (Fig. 1), which is required for the generation of pre-autophagosome structures and autophagy initiation (He and Klionsky, 2009; Mizushima, 2010). In yeast, mammals, and flies, ATG1 function is controlled by phosphorylation events mainly through the Target of Rapamycin (TOR) kinase (Kamada et al., 2000; Chang and Neufeld, 2009; Jung et al., 2009; Fig. 1), a central controller of cell growth in all eukaryotes (Wullschleger et al., 2006) functionally conserved in plants and algae (Menand et al., 2002; Crespo et al., 2005; see below). An important upstream positive regulator of autophagy in mammalian cells is the evolutionarily conserved AMPK (for AMP-activated protein kinase) protein, which stimulates autophagic processes by inhibiting mTORC1 signaling at the level of Tuberous Sclerosis Complex2 (TSC2; Inoki et al., 2003) in response to energy (ATP) level limitation (Alers et al., 2012). It has recently been

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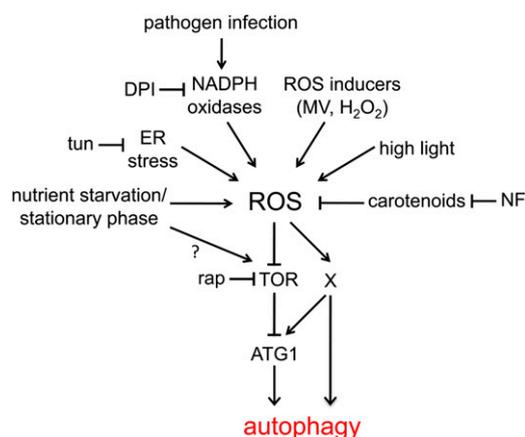


Figure 1. Proposed model of autophagy regulation by ROS in plants and algae. The intracellular level of ROS is modulated by different signals, including high-light stress, nutrient limitation, ER stress, or pathogen infection. ROS ultimately lead to ATG1 activation and hence autophagy induction either via TOR signaling or a TOR-independent mechanism, denoted here as X. This unknown pathway might control autophagy through ATG1 regulation, as reported in mammalian cells for AMPK (SnRK1 in plants), or by a different mechanism as described for the ATG4 protease. See text for details. DPI, Diphenylene iodonium; NF, norflurazon; rap, rapamycin; tun, tunicamycin.

shown that in addition to this indirect pathway, AMPK regulates autophagy by direct phosphorylation of Ulk1 (the mammalian ATG1 homolog) in coordination with mTORC1 (Kim et al., 2011).

The evolutionary conservation of structural and regulatory ATG proteins in photosynthetic eukaryotes strongly suggests the presence of similar regulatory mechanisms of autophagy in these organisms. In agreement with this hypothesis, TOR has been shown to inhibit autophagy in plants and algae (Liu and Bassham, 2010; Pérez-Pérez et al., 2010), whereas ATG1 activity appears to participate in autophagy regulation in plants (Suttangkakul et al., 2011). Nevertheless, our current knowledge of the molecular mechanisms and signals by which TOR and ATG1 control autophagy in photosynthetic organisms is still very limited. The purpose of this review will be to highlight recent findings connecting redox signals to the control of autophagy in plants and algae and to discuss how regulatory components in the autophagy process integrate these stress signals based on our current knowledge from other systems.

EXPERIMENTAL EVIDENCE CONNECTING REACTIVE OXYGEN SPECIES AND AUTOPHAGY IN PLANTS AND ALGAE

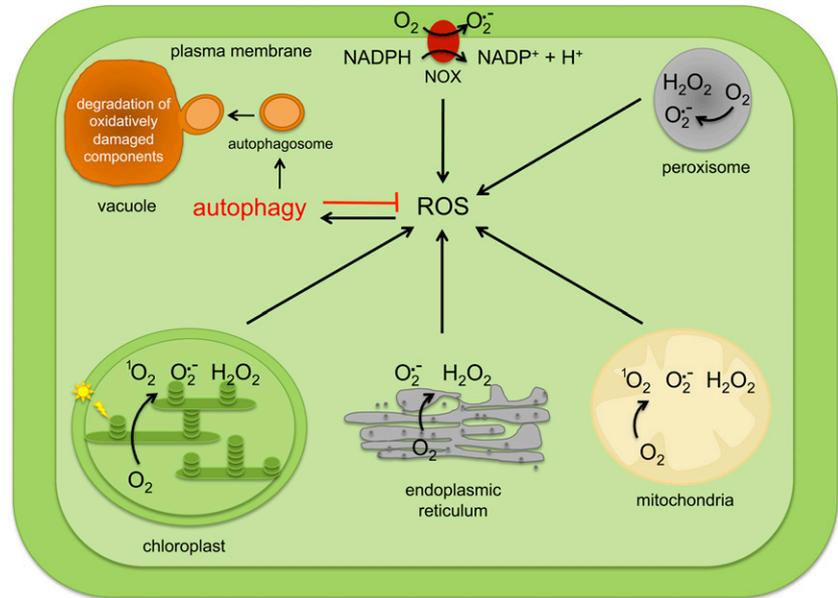
Excessive ROS may cause irreversible oxidative damage to proteins, lipids, and nucleic acids and activate signaling pathways ultimately leading to cell death (Apel and Hirt, 2004). However, at low levels, ROS participate in prosurvival mechanisms, acting as second messengers that transmit initial stress signals allowing cells to react and adapt to different environmental cues (Mittler et al., 2011). The chloroplast is

known to be one of the main sources of ROS in plants and algae. Superoxide anions ($O_2^{\bullet-}$) are generated as by-products of photosynthetic electron transport and readily converted into hydrogen peroxide (H_2O_2) inside the chloroplast through chemical and enzymatic reactions. Singlet oxygen (1O_2) and hydroxyl radicals are also produced during photosynthesis and can cause oxidative damage. In addition to the chloroplast, ROS are produced in plant cells in mitochondria, peroxisomes, and at the plasma membrane by NADPH oxidases (NOX; Fig. 2). Among different types of ROS generated in the chloroplast, H_2O_2 is the most stable molecule and diffuses across membranes, in striking contrast to $O_2^{\bullet-}$ and hydroxyl radicals, which are not usually released from functional chloroplasts. The highly reactive 1O_2 is generated predominantly at the reaction center of PSII, but despite its short half-time (about 200 ns; Krieger-Liszkay, 2005), it can play a role in signaling either by diffusion outside the thylakoid membrane (Fischer et al., 2007) or through second messengers, such as the carotenoid oxidation product β -cyclocitral (Ramel et al., 2012). Indeed, besides its toxic effects, 1O_2 was shown to trigger, in plants and algae, different signaling cascades leading to programmed cell death (Wagner et al., 2004) or to acclimation (Ledford et al., 2007) through regulation of the expression of specific genes (op den Camp et al., 2003; Gadjev et al., 2006; Fischer et al., 2009). Among ROS, H_2O_2 best fulfills the requirements of being a second messenger, as its stability, its membrane permeability, its reactivity that provides specificity for the oxidation of thiols, and its enzymatic production and degradation that provide specificity for time and place are required for signaling (Forman et al., 2010). H_2O_2 is recognized as an important signaling molecule in a wide range of organisms, including plants. H_2O_2 regulates numerous processes such as cell division, differentiation, and growth as well as apoptosis and plays a major role in the control of plant development and adaptation to biotic and abiotic stresses (Foyer and Noctor, 2009). Recent studies in photosynthetic organisms described the activation of autophagy in response to several stimuli that increase ROS generation, regardless of the origin and location of ROS production in the cell (Xiong et al., 2007; Liu et al., 2009; Pérez-Pérez et al., 2010, 2012).

Induction of Autophagy by H_2O_2 and Methylviologen

Treatment of *Arabidopsis* (*Arabidopsis thaliana*) plants with H_2O_2 results in severe oxidative stress and leads to the induction of autophagy (Xiong et al., 2007). Under these conditions, different types of irreversible oxidation of proteins occur, including carbonylation, sulfinic/sulfonic acid formation, or Tyr nitration. The finding that mutant plants defective in autophagy are hypersensitive to H_2O_2 and accumulate carbonylated proteins demonstrated that this degradative process is required for the cellular adaptation to oxidative stress. In close agreement, it has been shown that *atg2* and *atg5* *Arabidopsis* mutants accumulate high levels

Figure 2. Different ROS sources control autophagy in plants and algae. ROS can be generated by plasma membrane-localized NOX and different organelles, including chloroplast, mitochondria, peroxisome, and ER. Excess ROS then induce autophagy, which contributes to down-regulate ROS production and remove damaged cellular components. See text for details.



of H₂O₂ (Yoshimoto et al., 2009). Similar to H₂O₂, treatment of plants with the ROS-producing agent methylviologen (MV) also triggers autophagy (Xiong et al., 2007), although the molecular mechanism by which MV generates ROS differs from H₂O₂. MV is able to intercept electrons from PSI in the chloroplast or from the respiratory electron transport chain in the plant mitochondria and then reacts with oxygen to produce O₂^{•-}, which in turn is rapidly converted to H₂O₂. Therefore, MV generates ROS in the chloroplast and/or in the mitochondria in plants and may induce autophagy by oxidative damage caused in different organelles. Treatment of cells of the green alga *Chlamydomonas reinhardtii* with H₂O₂ or MV also results in autophagy activation (Pérez-Pérez et al., 2010, 2012). *Chlamydomonas* can grow in the dark using acetate as a carbon source. Therefore, contrary to plants, darkness does not induce autophagy in this alga (Pérez-Pérez et al., 2010). The ability of *Chlamydomonas* to grow heterotrophically provided a unique advantage to dissect the origin of redox signals in response to oxidative stress. As a ROS, H₂O₂ similarly induces autophagy under light or dark conditions, whereas MV requires photosynthetic electron flow to produce ROS and to trigger this catabolic process (Pérez-Pérez et al., 2012), suggesting that ROS generated in the chloroplast may signal to the activation of cytoplasmic autophagic machinery. Nevertheless, treatment of *Chlamydomonas* cells with MV in the dark also resulted in weak autophagy activation likely due to ROS production in the mitochondria (Pérez-Pérez et al., 2012).

NOX in Autophagy Regulation

Plant NOX, also known as respiratory burst oxidase homologs or Rboh, are localized at the plasma membrane

and catalyze the production of O₂^{•-} (Suzuki et al., 2011a). These ROS-generating enzymes play a central role in the redox network in plants by integrating ROS with other signaling pathways and mediate many processes such as the hypersensitive response by pathogen infection or systemic signaling in response to biotic and abiotic stresses (Suzuki et al., 2011a). NOX activity has been involved in autophagy regulation in plants (Liu et al., 2009). Pharmacological inhibition of NOX with the chemical inhibitor diphenylene iodonium revealed that induction of autophagy by nutrient (nitrogen or carbon) limitation or salt stress requires NOX activity. Therefore, ROS generated by NOX appear to be required for the activation of autophagy in response to nutrient and salt stress, emphasizing a possible role of ROS in the control of autophagy in plants. Interestingly, osmotic stress, which also triggers autophagy in plants, does not seem to be mediated by NOX (Liu et al., 2009). This remarkable finding indicates that autophagy is differentially regulated under starvation, salt, and osmotic stresses, and while autophagy in nutrient-starved and salt-stressed plants is regulated by a NOX-dependent pathway involving ROS, induction of this process by osmotic stress is regulated by a NOX-independent pathway. Autophagy has been analyzed in several NOX single mutants (Liu et al., 2009), but the presence of multiple *Rboh* genes in the *Arabidopsis* genome (Foreman et al., 2003) may complicate the identification of the specific NOX isoform(s) responsible for autophagy regulation in plants.

Pathogen infection is a potent trigger of autophagy in plant cells (Liu et al., 2005). ROS might mediate autophagy induction in response to this biotic stress. Upon infection, ROS are generated at the plasma membrane by NOX, although other organelles such as mitochondria, chloroplasts, and/or peroxisomes maintain enhanced ROS production (Torres et al., 2005,

2006). A recent study in *Arabidopsis* revealed that NOX (in particular AtRbohF) may also play an important role in coupling intracellular ROS generated by these organelles to downstream changes in redox state and cell death associated with salicylic acid signaling (Chaouch et al., 2012). Given the potential role of ROS in the control of autophagy, NOX might participate in the activation of this catabolic process during the initial stage of pathogen infection. This hypothesis could be tested with available tools and reagents, including NOX inhibitors, *rboh* mutants, or established autophagy markers in plants.

Photooxidative Damage Triggers Autophagy

A link between ROS generation in the chloroplast, photooxidative damage, and autophagy activation has recently been shown in *Chlamydomonas*. The analysis of carotenoid-deficient mutants revealed that the absence of photoprotection leads to increased levels of ROS in the chloroplast and a pronounced increase in autophagic activity (Pérez-Pérez et al., 2012). The *Chlamydomonas lts1-204* mutant lacks the phytoene synthase gene, one of the first enzymes in the carotenoid biosynthetic pathway, and therefore cannot grow in the presence of light due to the total absence of protective carotenoids (Inwood et al., 2008). This mutant exhibits high levels of autophagy even in the dark, which are further induced when cells are shifted to light (Pérez-Pérez et al., 2012). Carotenoid depletion can also be achieved through the chemical inhibition of phytoene synthase with the herbicide norflurazon (Sandmann and Albrecht, 1990). The finding that treatment of *Chlamydomonas* cells with norflurazon triggered autophagy in the light but not in the dark despite efficient carotenoid depletion under both conditions suggested that autophagy is associated with photooxidative damage caused by light-driven ROS production. Supporting this model, a transient activation of autophagy has been observed in wild-type *Chlamydomonas* cells subjected to high-light stress (Pérez-Pérez et al., 2012). A further demonstration came from the observation that the *npq1 lor1* *Chlamydomonas* mutant, which lacks specific photoprotective carotenoids and therefore is high light sensitive (Niyogi et al., 1997), displays permanent high levels of autophagy when exposed to this stress (Pérez-Pérez et al., 2012).

As discussed above, NOX participate in the induction of autophagy upon nutrient limitation and salt stress in plants. However, it is currently unknown if NOX might play a similar role in the redox regulation of autophagy in algae. Despite initially being proposed to be absent in algal genomes (Mittler et al., 2011), an improved annotation of the *Chlamydomonas* genome has recently allowed the identification of two NOX homologs in this organism (Anderson et al., 2011; Pérez-Pérez et al., 2012), although it remains to be experimentally confirmed whether these algal proteins indeed have

NOX activity. The effect of diphenylene iodonium on the activation of autophagy upon photooxidative damage has been tested in carotenoid-depleted *Chlamydomonas* cells, and interestingly, autophagy was partially, but not fully, suppressed with this inhibitor (Pérez-Pérez et al., 2012). This finding established a functional link between ROS and autophagy induction in *Chlamydomonas* and strongly suggested that plasma membrane-localized NOX participates in autophagy activation either directly or indirectly by contributing to the buildup of cellular ROS levels under stress conditions. It is possible, therefore, that NOX operate to amplify redox signals generated in response to other stresses in *Chlamydomonas*, similar to the feedback amplification of calcium signals reported for plant Rboh (Ogasawara et al., 2008; Takeda et al., 2008).

ROS IN STARVATION-INDUCED AUTOPHAGY

Autophagy has been defined as a degradative process induced by nutrient limitation for the recycling of intracellular material that might be used as building blocks to temporarily overcome the absence of nutrients. It is well established that nutrient starvation triggers autophagy by down-regulating TORC1 signaling in yeast and metazoans (see below). However, accumulating studies performed in different systems indicate that nutrient limitation also increases ROS production, which in turn may stimulate autophagy. Suzuki et al. (2011b) demonstrated that yeast mutant cells deficient in autophagy accumulate ROS to a higher extent than wild-type cells during nitrogen starvation and that ROS are a major factor contributing to the impaired respiratory function that leads to cell death. The authors proposed that autophagy is required during starvation for the maintenance of mitochondria function that is important for cell survival. As discussed above, Liu et al. (2009) showed that inhibition of NOX activity in plants avoided autophagosome formation in response to Suc or nitrogen starvation, strongly suggesting that ROS may function as signaling molecules to induce autophagy during nutrient stress. In mammalian cells, Scherz-Shouval et al. (2007) demonstrated that H₂O₂ produced in mitochondria serves as a signaling molecule during starvation-induced autophagy. Moreover, the accumulation of ROS appears to be essential for autophagy induction in nutrient-starved cells (Scherz-Shouval et al., 2007). Taken together, these findings indicate that the absence of nutrients is a primary signal leading to autophagy activation in eukaryotes, but this stress signal is tightly associated with the production and accumulation of ROS.

DO ROS MEDIATE AUTOPHAGY ACTIVATION IN ENDOPLASMIC RETICULUM STRESS?

The accumulation of un/misfolded proteins in the endoplasmic reticulum (ER) is a potent stress signal that induces the expression of chaperones and proteins

required for the reestablishment of cell homeostasis. This signaling process is defined as the unfolded protein response (UPR) and involves the activation of systems for protein quality control in the ER that function to prevent the accumulation of misfolded proteins (Ron and Walter, 2007). As part of the cellular response to ER stress, misfolded proteins are translocated into the cytosol, tagged with ubiquitin, and then degraded via the proteasome, a process that is known as ER-associated degradation (Meusser et al., 2005). ER stress is also a strong inducer of autophagy, and this signal is conserved from yeast to mammals (Yorimitsu et al., 2006). The use of ER stressors such as tunicamycin, an inhibitor of *N*-glycosylation, effectively activates an autophagic response similar to other stresses. In photosynthetic eukaryotes, a strong induction of autophagy by ER stress has been shown in *Chlamydomonas* (Pérez-Pérez et al., 2010), and based on the high conservation of both autophagy and ER stress responses, it is tempting to speculate that a similar link would exist in higher plants.

Given the essential role of disulfide formation in the folding and assembling of secretory and membrane proteins, protein folding in the ER is considered as a highly redox-dependent process. Several studies have established an association between ER stress and ROS generation (Malhotra and Kaufman, 2007; Rutkowski and Kaufman, 2007), and the molecular mechanisms by which ROS are produced during UPR have been thoroughly discussed in a recent review (Santos et al., 2009). These pathways include oxidoreductases present in the ER lumen such as Ero1, which can generate H₂O₂, although mitochondrial ROS and NOX have also been linked to ROS generation during ER stress. Is it possible that ER stress-generated ROS trigger or modulate autophagy? In response to ER stress, cells may initiate an autophagic process to remove unfolded proteins and harmful molecules, including ROS, or to counterbalance ER expansion caused by UPR during the recovery period, as proposed in yeast (Bernales et al., 2006), contributing to enhanced cell survival. Further studies will be required to determine whether ROS production is a main regulatory mechanism in ER stress-induced autophagy.

DEGRADATION OF ROS-GENERATING ORGANELLES VIA AUTOPHAGY

All organisms have evolved different ROS-scavenging systems (i.e. mechanisms controlling ROS accumulation). In plants and algae, enzymatic and nonenzymatic mechanisms have been reported for ROS scavenging, some of which are unique to these organisms due to the presence of chloroplasts as ROS factories (Foyer and Noctor, 2009). Nonenzymatic ROS-scavenging systems include potent antioxidants such as ascorbate and glutathione as well as membrane-localized carotenoids and tocopherols, which are exclusive to plants and algae. These antioxidant molecules are a major line of

defense against H₂O₂ and ¹O₂ produced in the chloroplast. Enzymatic ROS-scavenging mechanisms include catalase, heme peroxidases such as ascorbate peroxidase for H₂O₂ removal and superoxide dismutase for O₂^{•-} conversion to O₂ and H₂O₂, and thiol-based enzymes such as glutathione peroxidases and peroxiredoxins for diverse peroxides including H₂O₂ (Foyer and Noctor, 2009). Plants and algae can activate several defense systems simultaneously for the efficient scavenging of different ROS, but in some conditions, excess ROS can still be generated, causing massive damage in the cell. In this case, more aggressive mechanisms must be induced in the cell in order to remove damaged components and maintain ROS under control.

As discussed above, autophagy is a primary defense mechanism to degrade oxidized molecules, and it may also function to remove ROS-generating organelles. In yeast, ROS production during starvation or the postlog phase under respiratory conditions may contribute to the selective removal of mitochondria by autophagy, a process known as mitophagy (Kanki et al., 2009; Okamoto et al., 2009). In agreement with this model, the ROS scavenger *N*-acetyl-Cys is able to reduce mitophagy activity in yeast (Okamoto et al., 2009). Catalase, an important H₂O₂ scavenger, is localized in peroxisomes, and the specific degradation of this organelle by autophagy has been reported in several yeast species and mammals, in a process called pexophagy (Sakai et al., 2006). In plants, peroxisomes may play an important role in redox signaling and in the control of redox homeostasis, since they constitute a major source of H₂O₂ but can also produce O₂^{•-} radicals or nitric oxide (del Río et al., 2002; Foyer et al., 2009; del Río, 2011). Pexophagy has not yet been demonstrated in plants; therefore, the physiological role of peroxisome degradation and its implication in redox signaling remains unclear. The chloroplast is a primary source of ROS in plants and algae, and it may be targeted for degradation in a process called chlorophagy. Degradation of chloroplast material through autophagic processes has been reported in plants under carbon-limited conditions caused by darkness (Ishida et al., 2008; Wada et al., 2009; Izumi et al., 2010). Chloroplast degradation occurs via Rubisco-containing bodies, small spherical bodies containing chloroplast stroma that can be detected in the cytosol (Ishida et al., 2008). In addition to Rubisco-containing bodies, degradation of whole chloroplasts has been described in senescent plants (Wada et al., 2009). However, there are many degradation pathways for chloroplast material, including autophagy and proteolytic processes, but autophagy does not appear to be the main pathway, since Rubisco degradation is not compromised in autophagy-deficient plants (Wada et al., 2009). In close agreement, no direct correlation between autophagy activation and Rubisco degradation has been found in *Chlamydomonas* upon photooxidative damage (Pérez-Pérez et al., 2012).

Overall, autophagy may regulate ROS production by the degradation of specific ROS-generating organelles,

but this mechanism remains to be fully characterized, especially in plants.

TARGETS FOR REDOX REGULATION OF AUTOPHAGY

Based on experimental evidence discussed in the former sections, autophagy is possibly modulated by redox signals (Fig. 1) through regulatory mechanisms that might be conserved from yeast and algae to mammals and plants. But how are redox inputs perceived and transmitted to the autophagic machinery? So far, only a single target has been proposed to integrate redox signals for autophagy regulation, the ATG4 protease. However, given the complexity of this catabolic process, other regulatory components, such as the ATG1 and TOR kinases, might also participate in redox signaling.

The TOR-ATG1 Pathway

The Ser/Thr kinase ATG1 is an essential component of autophagy in all systems, and its catalytic activity is required for the regulation of the process at the initiation level (Nakatogawa et al., 2009; Mizushima, 2010). Originally identified in yeast (Tsukada and Ohsumi, 1993), ATG1 is widely conserved through evolution, and orthologs have been described in lower and higher eukaryotes (Meijer et al., 2007). To perform its regulatory function, ATG1 associates with other ATG proteins, including ATG13, which stimulates the kinase activity of ATG1 and is also evolutionarily conserved (Kamada et al., 2000; Hosokawa et al., 2009). In yeast, the activity of the ATG1/ATG13 complex is regulated by phosphorylation events on both proteins that are promoted by at least three different signaling pathways: TORC1, PKA (for cAMP-dependent protein kinase), and AMPK (Mizushima, 2010; Alers et al., 2012). These kinases differently modulate the interaction strength between ATG1 and ATG13 and, hence, the kinase activity of ATG1. While phosphorylation of ATG1 and/or ATG13 by TORC1 and PKA results in autophagy inhibition (Nakatogawa et al., 2009; Mizushima, 2010), AMPK signaling stimulates this degradative process (Alers et al., 2012).

The ATG1/ATG13 kinase complex as well as TORC1 and SnRK1 (plant AMPK) signaling pathways are conserved in the model plant *Arabidopsis* (Robaglia et al., 2012). Possible orthologs of *ATG1* and *ATG13* genes are present in the *Arabidopsis* genome, and their products appear to regulate autophagy in plants (Suttangkakul et al., 2011). However, the signaling pathways controlling ATG1/ATG13 function in plants remain unknown. The TOR and SnRK1 kinases have a central role in nutrient and energy sensing in photosynthetic organisms (Robaglia et al., 2012), and TORC1 signaling has been shown to regulate autophagy in plants and algae. A decrease in TOR function by reduced *AtTOR* transcription in *Arabidopsis* or rapamycin

treatment in *Chlamydomonas* resulted in a pronounced increase of autophagic activity in both systems similar to the one observed in nutrient-starved cells (Liu and Bassham, 2010; Pérez-Pérez et al., 2010). These findings underscored a prominent role of TOR in the control of autophagy in photosynthetic organisms but do not exclude that other conserved pathways such as SnRK1 signaling may also participate in the regulation of this process, as described in other systems.

The question arises whether the TOR pathway might perceive redox signals in photosynthetic organisms to regulate autophagy (Fig. 1). TOR integrates nutritional and energy inputs to coordinately promote cell growth (Wullschleger et al., 2006), and increasing evidence indicates that TOR may also be linked to redox signaling in lower and higher eukaryotes. Indeed, first, TOR is a key controller of mitochondrial function (Schieke and Finkel, 2006), which, as discussed above, is tightly connected to redox metabolism. Second, upstream regulators of TOR such as AMPK or TSC1/2 integrate redox signals via p53-regulated sestrins (Budanov, 2011). Third, there are multiple studies suggesting that ROS can both positively and negatively regulate TOR function (Blagosklonny, 2008; Budanov, 2011), but, at least for autophagy regulation, ROS mainly leads to TOR inactivation and hence autophagy activation (Alexander et al., 2010). TORC1 signaling is conserved in plants (Deprost et al., 2005, 2007; Mahfouz et al., 2006; Moreau et al., 2012) and algae (Crespo et al., 2005; Díaz-Troya et al., 2008a, 2011), but the absence of some key upstream regulators of TORC1, such as the TSC1/2 complex, strongly suggests that this signaling pathway might be differently regulated in these organisms. Without a reliable TORC1 substrate like ATG1 or ATG13 that could be used to specifically monitor the regulation of autophagy by this pathway in plants and algae, it might be premature to place ROS upstream of TOR for the control of autophagy. However, based on the proven role of TOR in autophagy regulation in photosynthetic organisms, together with the evident cross talk between redox signaling and TOR reported in other systems, it is tempting to speculate that, at least under some stress conditions, ROS might down-regulate TOR activity, which in turn would result in ATG1 activation and autophagy induction in plants and algae (Fig. 1).

ATG4 Integrates Redox Signals to Control Autophagy

At present, mammalian ATG4 is the only ATG protein whose activity has been shown to be a target for redox regulation (Scherz-Shouval et al., 2007). As discussed above, ATG4 has an essential function in autophagy. This Cys protease plays a dual role in autophagosome formation. On the one hand, ATG4 processes the C terminus of newly synthesized ATG8 (Kirisako et al., 2000), a first crucial step for the binding of ATG8 to PE by the lipidation system. ATG4 also delipidates ATG8 to recycle it from the autophagosome

membrane (Kirisako et al., 2000). The first function of ATG4 has been extensively studied; however, the delipidation role remains unclear. Recently, it has been proposed in yeast that the lipidation system produces constitutively and nonselectively ATG8-PE and that ATG4 acts to recycle ATG8-PE generated on inappropriate membranes to maintain a reservoir of unlipidated ATG8 that is required for autophagosome formation (Nakatogawa et al., 2012).

The molecular mechanism by which the lipidation and delipidation activities of ATG4 are regulated is not well understood. It has been proposed that H₂O₂ can inactivate mammalian ATG4 by oxidizing its regulatory Cys residue to prevent the processing of ATG8, whereas reducing conditions would result in ATG4 activation (Scherz-Shouval et al., 2007). This interesting model still leaves some open questions that would need to be answered to get a general overview about the *in vivo* regulation of ATG4 by redox signals. For instance, it is unknown whether processing and delipidating activities of ATG4 are differently regulated or simply separated in time and/or space, since ATG8 processing takes place in newly synthesized protein and is required for autophagosome formation, whereas delipidation has to happen when autophagy is not necessary anymore and ATG8 needs to be recycled. Access of ATG4 substrates to the active site of the enzyme also might constitute another regulatory mechanism of ATG4 activity, since structural studies performed with the human protease indicate that binding of ATG8 to ATG4 triggers large conformational changes in a regulatory loop and the N-terminal tail of the protein, allowing ATG8 access to the catalytic site (Satoo et al., 2009). These studies have been performed with human ATG4, and it remains to be tested whether the proposed redox regulation of this protein is conserved in other systems.

Two homologs of the *ATG4* gene have been identified in the *Arabidopsis* and maize (*Zea mays*) genomes (Yoshimoto et al., 2004; Chung et al., 2009), and their deletion in *Arabidopsis* results in a complete blockage of the autophagy process due to the inability of ATG8 isoforms to be modified (Yoshimoto et al., 2004), demonstrating that plant ATG4 is essential in autophagy. A single *ATG4* gene that participates in ATG8 processing is present in *Chlamydomonas* (Díaz-Troya et al., 2008b). Supporting this theory, ATG8 cleavage is catalyzed by an iodoacetamide-sensitive protease activity, which demonstrates the participation of Cys(s) in the control of this process (Pérez-Pérez et al., 2010). The high evolutionary conservation of autophagy, including both structural and regulatory elements, suggests that ATG4 might also be a central integrator of redox signals in photosynthetic organisms.

PERSPECTIVE

In ROS-dependent signaling pathways, protein thiols play a central role, as they couple the changes in

the intracellular redox state to biochemical responses. In response to ROS, redox-sensitive Cys residues of proteins undergo a diverse spectrum of reversible posttranslational modifications (Zaffagnini et al., 2012). Indeed, Cys residues can undergo different states of oxidation such as sulfenic, sulfinic, and sulfonic acid but also protein disulfide bridges (intramolecular or intermolecular), S-thiolation (mainly glutathionylation), or nitrosylation. These posttranslational redox modifications are mainly under the control of two types of ubiquitous disulfide oxidoreductases: thioredoxins and glutaredoxins. Therefore, the mechanisms underlying the induction of autophagy by ROS likely involve one or several target proteins that undergo ROS-triggered redox posttranslational modifications affecting their activity and resulting in the induction of autophagy. In mammals, this type of redox modification appears to control the activity of ATG4, but the Cys residues involved are not conserved, suggesting that this mechanism is not ubiquitous and that other targets remain to be identified. These target proteins may be known components of the autophagic machinery, like ATG4, or unknown regulators yet to be discovered. Identifying these ROS-controlled autophagy regulators and delineating the underlying molecular mechanism will likely represent a major challenge in the field for coming years.

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