REVIEW

Hydrogen sulfide action in the regulation of plant autophagy

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Hydrogen sulfide is a signalling molecule with a well-established impact on both plant and animal physiology. Intense investigation into the regulation of autophagy by sulfide in *Arabidopsis thaliana* has revealed that the post-translational modification of persulfidation/S-sulfhydration plays a key role. In this review focused on plants, we discuss the nature of the sulfide molecule involved in the regulation of autophagy, the final outcome of this modification and the persulfidated autophagy proteins identified so far. A detailed outline of the actual knowledge of the regulation mechanism of the autophagy-related proteins ATG4a and ATG18a from Arabidopsis by sulfide is also included. This information will be instrumental for furthering research on the regulation of autophagy by sulfide.

Keywords: abscisic acid; Arabidopsis; ATG18a; ATG4a; ER-stress; hydrogen sulfide; persulfidation; polysulfides; post-translational modification; S-sulfhydration

The chemical compound hydrogen sulfide (H_2S) is a colourless gas with a characteristic unpleasant odour that in nature is part of volcanic gases, hot springs and rock salts. It is very abundant in natural gases and is a subproduct of crude oil processing. Due to its chemical characteristics, H_2S is a highly toxic molecule that is hazardous to the environment and life. However, in low-oxygen environments, microorganisms base their vital cycle on H_2S [1], and the biochemistry of H_2S was crucial in primeval Earth ecosystems, thus being an early molecule in biological systems.

The toxicity of H_2S in living organisms stems from its inhibitory effect on complex IV of the mitochondrial respiratory chain, which hampers cell respiration, similar to other gases such as carbon monoxide (CO) and nitric oxide (NO) [2]. However, CO and NO at low non-toxic concentrations regulate numerous cellular processes and are considered signalling molecules that act as physiological gasotransmitters [3]. Likewise, H_2S shows the same duality (toxic vs. signalling molecule) and is currently recognised to be as relevant as CO and NO in animal systems, and as NO and hydrogen peroxide (H_2O_2) in plants [4,5]. Furthermore, the conception of H_2S as a biological molecule present in mammalian tissues dates back to the last century, and its endogenous production and signalling function were first established in the nervous system in the late 90s [6]. Similarly, in plants, H_2S is a well-established intermediate of photosynthetic assimilatory sulfate reduction [7].

Therefore, hydrogen sulfide is endogenously produced by mammalian and plant cells through different enzymatic reactions, with plant cells having different subcellular sources of H_2S [8]. The main source from a

Abbreviations
ABA, abscisic acid; CO, carbon monoxide; ER, endoplasmic reticulum; H_2S, hydrogen sulfide; LC–MS/MS, liquid chromatography–tandem mass spectrometry; LST8, Lethal with Sec Thirteen protein 8; PE, phosphatidylethanolamine; PTM, protein post-translational modification; ROS, reactive oxygen species; SnRK1, Snf1-related protein kinase 1; NO, nitric oxide; PTM, post-translational modification; TOR, target of the rapamycin.

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quantitative point of view is the chloroplast, where sulfite is enzymatically reduced to sulfide by sulfite reductase involved in the photosynthetic sulfate assimilation pathway. Another source is the cytosol, where H_2S is produced from cysteine by different enzymatic activities such as L-cysteine and D-cysteine desulfhydrases, which catalyse the conversion of L- or D-cysteine, respectively, to sulfide, ammonia and pyruvate. Other enzymes that generate H_2S not only in the cytosol but also in the chloroplast and mitochondria are NifS-like proteins that transform L-cysteine into alanine and elemental sulfur or sulfide. In mitochondria, β-cyanoalanine synthase is an enzyme that uses cysteine for the detoxification of cyanide and produces H_2S. Whether and how these different sources of sulfide are integrated or even if one prevails over the others when regulation of a specific plant process by H_2S takes place is completely unknown.

Hydrogen sulfide is weakly acidic and, in aqueous solutions, it dissociates into the hydrogen cation H^+ and the hydrosulfide anion HS^-, which subsequently can decompose into H^+ and the sulfide anion S^{2-}, with pKa values of 6.9 and > 12 respectively. Therefore, under physiological pH conditions (pH 7.4), one-third of the total H_2S pool is in the form of neutral gas (H_2S) and two-thirds are in the form of HS^-, whereas S^{2-} is not present [9]. Hereinafter, H_2S refers to both the neutral and anionic forms. Only the fraction of neutral H_2S can freely pass biomembranes to distribute the sulfide species within the cells. It has experimentally been shown that pH has no effect on H_2S diffusion through one to two membrane layers, although increasing distance that H_2S has to diffuse and the membrane layers that it must cross would impact the process [10].

Intensive research on H_2S carried out in both animal and plant systems in the last years has identified a huge number of physiopathological processes regulated by sulfide. In plants, H_2S has been demonstrated to play a regulatory role in the responses to different adverse environmental conditions, mainly abiotic stresses. Thus, H_2S induces positive effects on a variety of plant species when they face a threatening condition, such as drought, hypoxia, flooding, salinity, heat, chilling, different metals and ion deficiency, and the number of reports where H_2S benefits the plant against new adverse conditions continuously increases [5,11,12] so that it is now evident that H_2S improves plant resilience, often affecting different developmental processes and/or inducing antioxidative defences [8]. Likewise, H_2S regulates plant processes essential for the whole life cycle, such as different developmental programs, directly or through crosstalk with other signalling molecules and hormones [4,13]. Two processes regulated by H_2S have been clarified down to the underlying molecular mechanisms, thanks to extensive investigation performed in *Arabidopsis thaliana*: the H_2S regulation of abscisic acid (ABA)-dependent stomatal movement, described elsewhere, and of autophagy, the main topic of this review.

Autophagy is a considerably well-known eukaryotic degradative pathway with a distinctive pro-survival role. Different types of autophagy have been described (reviewed elsewhere) and here we focus on macroautophagy (hereinafter referred to as autophagy), the best-studied plant autophagy type. This process is characterised by *de novo* synthesis of the autophagosome in which cytoplasmic constituents are sequestered, transported and released into plant vacuole for recycling. The core mechanism of autophagy is highly conserved in all eukaryotes studied so far, and the involved proteins are named autophagy-related proteins (ATG) [14,15]. Although autophagy was originally considered a non-selective process, it is well known that it can selectively degrade specific cargos by binding to selective receptors/adapters, which get recruited upon binding to different interacting motifs of autophagosome-anchored ATG8 protein [16–23]. Initially, plant autophagy was described as an important process in nutrient recycling, senescence and under nitrogen and carbon limitations [24]. Nowadays, it is established that autophagy occurs at basal levels to maintain homoeostasis, and also is induced by other development programs during the plant life cycle and by different abiotic and biotic stressors [20,25,26].

Autophagy is an essential process and, unsurprisingly, is tightly regulated at many different levels. Target of the rapamycin (TOR) complex is a key negative regulator of autophagy that is active when nutrients are sufficient to allow growth and gets inactivated under nutrient deprivation thereby enhancing autophagy activity. Energy sensor Snf1-related protein kinase 1 (SnRK1), which inhibits the TOR complex, and the TOR substrate ATG1 kinase complex are also regulators of autophagy [15,27] (details described elsewhere). Another important player in autophagy regulation are reactive oxygen species (ROS), which are involved in plant responses to many abiotic and biotic stress conditions. Under stress, increased ROS production causes the oxidation of proteins and organelle damage, triggering the degradation of non-functional cell components by autophagy. However, several ATG proteins are sensitive to the cellular redox state, as well as TOR and SnRK1. When ROS reach very high levels, these proteins can be oxidised, halting autophagy [25,28]. Here, we focus on a different regulator of autophagy, hydrogen sulfide, which inhibits

autophagy using specific molecular mechanisms that have been discovered in recent years.

Mechanism of hydrogen sulfide function

The molecular mechanism by which H_2S plays its role in regulating and signalling vital processes is based on the chemical characteristics and reactivity of this molecule. Its weak acid behaviour, its reductant nature and its metal binding capacity led to suggest different mechanisms of action [29]. As described above, H_2S also includes the anionic form HS^-, and both forms can interact with metal centres on protein and covalently modified. This mechanism has been well documented to be responsible for its inhibition of respiratory cytochrome c oxidase and its reaction with haemoglobin and myoglobin.

The interaction of H_2S with ROS and other biological oxidants has also been suggested based on the fact that the oxidation state of −2 of the sulfur atom is the lowest state, and therefore, H_2S can only oxidise. Although the direct reaction of H_2S with oxygen is thermodynamically disfavoured, other oxidants, such as hydroxyl ($HO^{\cdot}$) and superoxide ($O_2^{\cdot-}$) radicals, hydrogen peroxide (H_2O_2), nitrogen dioxide ($NO_2^{\cdot}$), peroxynitrite (ONOOH), and others, can oxidise H_2S [29]. In connection with the reductant nature of H_2S, in many cases, it has been suggested that H_2S alleviates oxidative stress by increasing the antioxidative defences of the plant under stress conditions. The interplay between ROS and H_2S has been extensively demonstrated [8,11,12].

Persulfidation/S-sulfhydration

The third mechanism of action of H_2S that was suggested and is currently well established and predominantly studied consists of the modification of cysteine residues in proteins, therefore transforming thiol groups (−SH) into persulfide groups (−SSH). This mechanism is known as persulfidation or as S-sulfhydration and is a very predominant protein post-translational modification (PTM), at least, in *Arabidopsis thaliana* [30,31]. This modification was first described in animal systems using a detection method for persulfidated proteins that was a modification of the biotin switch assay described for the detection of proteins by S-nitrosylation [32]. The same and other improved methods have been used in plants to identify protein persulfidation [30].

Change in a thiol group by a persulfide in a specific cysteine residue significantly affects protein function that can be caused by conformational or charge changes in protein structure, as suggested [33,34]. Persulfide residues are more nucleophilic and acidic and therefore more reactive than the thiols, and the effects on the protein function depend on the specific target protein. Consequently, different studies on specific proteins have shown that this PTM alters enzymatic activity, activating or inhibiting, binding capacity or intracellular location, and consequently causes important physiological outcomes [33–41].

Nature of the sulfide molecule involved in persulfidation

The nature of the persulfidating molecule that modifies the thiol group on proteins to generate the persulfidation is still under intense debate, and it has to be ascertained not only in plants but also in animal systems. As described above, chemically H_2S is a reductant and cannot react directly with the thiol group where the sulfur atom is in the same oxidation state. Therefore, different situations have been proposed regarding either the sulfide-donor molecule or the actual oxidation state of the cysteine residue to generate the persulfide group.

Thus, as persulfidating agents have been proposed polysulfides and persulfide molecules that contain sulfane, which refers to a sulfur covalently bonded to two or more sulfur atoms or to a sulfur atom and ionisable hydrogen [29]. It has been suggested that sulfide oxidation pathways that generate a series of reactive sulfur species, including persulfides and polysulfides, could be responsible for intracellular sulfide signalling through persulfidation [42]. In fact, in plants, exogenous polysulfide treatments are routinely used to induce the persulfidation of proteins *in vitro* and in plant tissues *in vivo* [33]. Moreover, a very different mechanism has been proposed that consists of direct incorporation of persulfidated cysteine proteins, due to the identification of prokaryotic and mammalian cysteinyl-tRNA synthetase [43].

Although H_2S cannot react with thiol, it is possible that its reaction with oxidised cysteine residues is possible, and this is a scenario that has recently been supported by different investigations. Therefore, H_2S can react with sulfenic acid (−SOH), nitrosothiols (−SNO) or other oxidised residues to generate persulfides, but the most thermodynamically favourable process is the reaction with sulfenic residues. Therefore, protein persulfidation should rely on the formation of protein sulfenylation [44,45]. In fact, a temporal dynamics of protein sulfenylation and persulfidation in different cell types have shown that persulfidation increases

following a phase-shifted curve after an increase in sulfenylation [46]. It has been proposed that after transient ROS production induced by developmental or stress signals, the level of sulfenylation increases prior to activation of sulfide-generating enzymes that induce an increase in protein persulfidation. Accordingly, many different plant studies have shown that, under stress situations, endogenous H_2S production occurs to combat oxidative stress due to ROS production [8]. Therefore, H_2S and H_2O_2 signal transductions should be interconnected [11].

In some way, this interconnection is in accordance with the initial proposed role of persulfidation consisting of preventing the overoxidation of cysteine residues. Under persistent oxidation stress, sulfinic (−SO2H) and sulfonic (−SO3H) motifs are formed that are irreversible, resulting in loss of protein function [46,47]. On the contrary, the persulfidated residues can react further with ROS generating different oxidised perthiol residues that are reduced to thiol by biological reductants [5,8,29], consequently protecting the proteins of excessive oxidation.

Regulation of non-selective and ER-selective autophagy by hydrogen sulfide

The initial clue to the regulation of autophagy by H_2S in plants came from the characterisation of Arabidopsis *des1* mutants defective in L-cysteine desulfhydrase DES1 located in the cytosol [48]. This enzyme catalyses the desulfuration of cysteine into sulfide plus ammonia and pyruvate, and was unequivocally established to be involved in the degradation of cysteine and the concomitant generation of H_2S in the cytosol [49]. Mutation in the *DES1* gene leads to premature leaf senescence that was demonstrated phenotypically, also by the induction of expression levels of senescence gene markers and by the generation of senescence-associated vacuoles in mesophyll protoplasts [48,50].

Further investigation in leaves from *des1* mutants demonstrated that DES1 deficiency promotes the accumulation and lipidation of the ATG8 protein, indicative of the induction of autophagy (Fig. 1). In addition, restoring the generation of H_2S in *des1* mutants, both by exogenous addition of NaHS as a source of sulfide or by gene complementation, rescues the induced autophagy phenotype. This reversion was also observed at the cellular and transcriptional levels [50]. All these data brought to light hydrogen sulfide as a signalling molecule in autophagy through negative regulation [51], and it was the starting point to deepen this topic.

Non-selective autophagy (bulk autophagy)

A very well-established condition that induces the non-selective autophagy process is the limitation of nutrients, and extensive research on this topic has been conducted in a variety of plant species [24,52]. To determine whether H_2S regulates autophagy independently of DES1, darkness-induced carbon starvation was imposed on wild-type Arabidopsis and *des1* mutants in the presence or absence of sulfide. The results showed that the induced characteristic phenotype traits, the ATG8 protein accumulation and its lipidation by carbon limitation were clearly reversed by exogenous sulfide application in both genetic backgrounds (Fig. 1). In addition, the same carbon limitation experiment was carried out in the presence of ammonia, another product of the enzymatic activity catalysed by DES1, and unlike sulfide no reversion was observed [50]. Therefore, this research reinforces the signalling role of H_2S, and not of the absence of functional DES1, in autophagy acting as a repressor, and in a way unrelated to sulfur limitation.

A different experimental system was established to study the role of H_2S and to determine its mechanism of action, consisting of roots subjected to nitrogen limitation from wild-type Arabidopsis plants expressing the GFP-ATG8a fusion protein. Both by confocal quantification of GFP-tagged autophagic bodies and by immunoblotting analysis of GFP, it was demonstrated that exogenous sulfide significantly repressed autophagy induced under nitrogen deprivation [53] (Fig. 1). Importantly, in this research, it was also demonstrated that the only inorganic sulfur-containing compound able to reverse autophagy is H_2S. A new experiment was performed, in an effort to determine the mechanism of action of sulfide, due to under stress condition, an increase in ROS levels causes the induction of autophagy and a role for sulfide alleviating the oxidative damage has been suggested. In this way, the nitrogen deprivation experiment was performed in the presence of well-known antioxidants, such as glutathione and ascorbic acid. The results showed that H_2S (NaHS as sulfide source) cannot scavenge ROS generated by nitrogen limitation, in contrast to these antioxidants that reduce them to undetectable levels. Furthermore, it was demonstrated that neither glutathione nor ascorbic acid inhibits autophagy induction to the extent observed for H_2S [53]. Therefore, this study reached the conclusion that the mechanism by sulfide represses autophagy is independent of the redox conditions.

A different study in which autophagy was induced by ABA treatment, a phytohormone that increases its concentration when a plant is subjected to abiotic

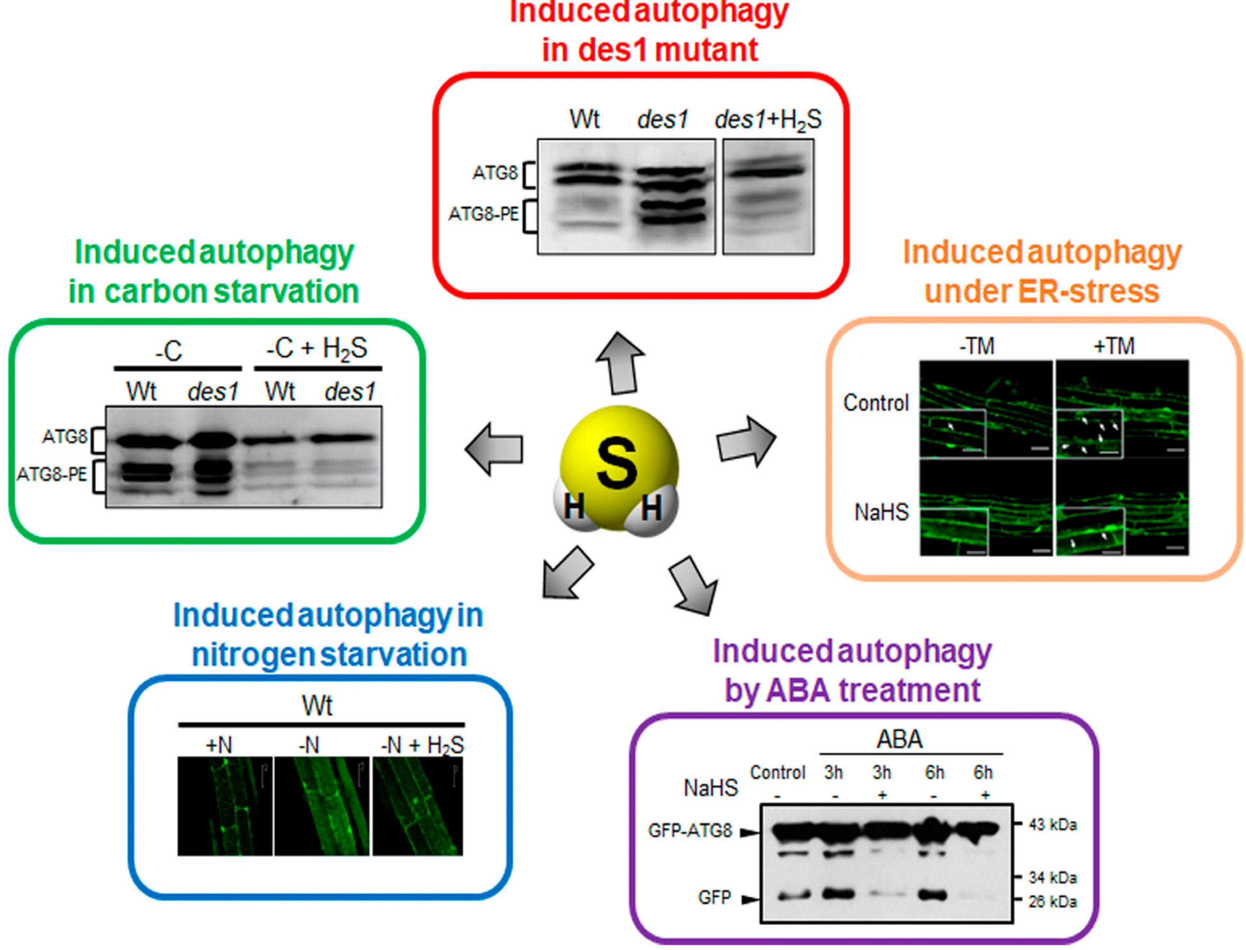

Fig. 1. Hydrogen sulfide negatively regulates autophagy induced by different situations. H_2S-mediated autophagy signalling has been demonstrated in des1 mutant plants [50], in stresses such as carbon [50] and nitrogen deprivation [53], ER-stress [34] and by ABA treatment [33]. 'Copyright (2021) National Academy of Sciences and copyright (2012, 2016, 2020) Oxford journals'.

stress conditions, also showed the repression of ABA-induced autophagy by NaHS (Fig. 1) [33]. Therefore, this research demonstrated that sulfide also plays a signalling function in the regulation of bulk autophagy induced by abiotic stress.

Collectively, all the described findings reach the conclusion that sulfide functions as a signalling molecule negatively regulating non-selective autophagy induced by stress in plant systems. This signalling role is unrelated to sulfur nutrition and is independent of ROS, and in all situations, the final outcome seems to be improving plant performance.

ER-selective autophagy

The endoplasmic reticulum (ER) is a highly complex organelle that is essential for cellular metabolism and signal transduction. It is mainly composed of membranes and tubules, where most of the proteins and lipids are synthesised in eukaryotic cells. The ER has a highly regulated dynamic turnover to adjust to cellular requirements. The ER may be stressed upon different stimuli such as hypoxia, high energy demand or accumulation of unfolded proteins [54].

To maintain homoeostasis in the cell and in the ER, eukaryotes have developed several mechanisms to control it and remove misfolded proteins. One of these mechanisms is ER-selective autophagy, named ER-phagy, and dysregulation of ER-phagy has been associated with various diseases [55,56]. In ER-phagy, specific receptors bind to cargoes, in a similar way as other selective autophagy, mediating the degradation of certain regions of ER *via* autophagy in lysosomes/vacuoles, using the core autophagy machinery. Several receptors for ER-phagy have already been deciphered in mammals, such as cell cycle progression

protein 1 (CCPG1) [57], family with sequence similarity 134 (FAM134B) [58], reticulon domain-containing proteins (RTN3) [59], the translocon component SEC62 [60], atlastin GTPase 3 (ATL3) [61] and testis-expressed protein 264 (TEX264) [62,63]. Three other receptors have been identified in yeast, Atg39 and Atg40 [64] and Epr1 [65]. Several studies in plants have led to deciphering two more receptors for ER-phagy, ATI1 and ATI2 [66], and more recently, a new receptor cross-kingdom has been described in plants, Arabidopsis Sec62 (AtSec62) [67], and the root hair defective3 (RHD3) receptor too [68]. In addition, mammalian C53 protein, also found in plants, has also been identified as an ER-phagy receptor, which does not behave as other receptors interacting with ATG8 directly through its AIM region, it interacts with ATG8 by a non-canonical AIM instead [69].

Although the role of H_2S that regulates ER-phagy needs to be further studied, quite recent publications have described the beneficial effect of H_2S inhibiting ER stress, improving cellular bioenergetics and preventing autophagic arrest [70]. Therefore, it has been shown the beneficial effect of sulfide alleviating various diseases, such as central nervous system degenerative diseases [71] and disruption of the blood–spinal cord barrier [72]. Unfortunately, studies on the role of H_2S in the regulation of ER-phagy in plants are scarce. However, it should be noted that H_2S has been shown to negatively regulate ER degradation after tunicamycin-induced ER stress [34] (Fig. 1). ER structures were observed within the vacuoles of Arabidopsis roots using GFP-HDEL plants when they were subjected to ER stress conditions by tunicamycin treatment. However, plants treated with H_2S, under ER stress, did not show ER degradation in vacuoles, demonstrating the negative regulation of sulfide in ER degradation by autophagy [34]. In addition, these authors demonstrated that the underlying mechanism was not due to ROS scavenging, which could cause ER stress alleviation. They also treated plants with ascorbic acid and glutathione under induction of ER stress by tunicamycin, but no significant decrease in autophagosomes was detected with these treatments, indicating that the effect observed with sulfide treatment was not due to its antioxidant properties [34].

Persulfidated autophagy-related proteins in Arabidopsis

The findings acquired in plant systems exhibit that the H_2S regulation of both bulk and selective autophagy is independent of its capacity to react with ROS, and therefore, the underlying mechanism of this regulation is not due to the reductant nature of H_2S. Profound investigation (described below) in which high-throughput proteomics was combined with labelling and enrichment methods, together with the characterisation of specific autophagy-related (ATG) proteins, brought out persulfidation as the underlying mechanism of sulfide signalling in plants. Very recently, persulfidation has also been demonstrated in the regulation of autophagy in animal systems, suggesting that it is a conserved mechanism in both eukaryotes [73].

Proteomic studies

The first proteomic analysis enabled the detection of endogenous persulfidated ATG proteins was performed on leaves of Arabidopsis wild-type and *des1* mutant plants grown under controlled conditions using the tag switch method [30]. More than 2000 persulfidated proteins were identified that comprise at least 5% of the entire Arabidopsis proteome. Bioinformatic analysis showed that persulfidated cysteines participate in various biological processes and new pathways were revealed in which this PTM is a new regulatory component [30]. Concerning ATG proteins, proteomic analysis on wild-type samples demonstrated the susceptibility of some autophagic core proteins, such as ATG18a, ATG3, ATG5 and ATG7, to persulfidation (Table 1). On the contrary, the proteomic analysis in *des1* mutants, with a reduced cytosolic H_2S content [50], only ATG18a was detected as a target for persulfidation. Therefore, this proteomic study showed for the first time that the mechanism of sulfide signalling of autophagy is the persulfidation of specific ATG proteins.

A new comparative and label-free quantitative proteomic analysis was developed in Arabidopsis roots under N-limitation, a well-established condition that induces non-selective autophagy, in order to detect additional persulfidated autophagy-related proteins [31]. More than 5000 proteins from root tissues were identified under both N-sufficient and N-starved conditions, resulting in a significant improvement of the Arabidopsis persulfidome, now comprising almost 13% of the entire annotated proteome for *Arabidopsis thaliana*. Bioinformatic analysis of these proteins based on their assigned functions revealed that the most abundant set corresponded to the general protein group, including those involved in protein degradation. Specifically, several key proteins of the autophagy process were identified, such as TOR1 and its effector proteins regulatory-associated protein of TOR1 (RAPTOR 1) and Lethal with Sec Thirteen protein 8

Table 1. Identified persulfidated proteins involved in autophagy process.

Protein name	AGI	Gene annotation	Reference
Autophagy-related protein 2	AT3G19190	ATG2	[31]
Autophagy-related protein 3 (APG3)	AT5G61500	ATG3	[30,31]
Cysteine protease ATG4a	AT2G44140	ATG4a	[31,33]
Cysteine peptidase family C54 protein	AT3G59950	ATG4b	[31]
Autophagy-related protein 5 (APG5)	AT5G17290	ATG5	[30,31]
Autophagy-related protein 7 (APG7)	AT5G45900	ATG7	[30,31]
Autophagy-related protein 11	AT4G30790	ATG11	[31]
Autophagy-related protein 13	AT3G49590	ATG13	[31]
Autophagy-related protein 18a	AT3G62770	ATG18a	[30,34]
Transducin/WD40 repeat-like superfamily protein	AT3G18140	LST8-1	[31]
Protein phosphatase 2A-2	AT1G59830	PP2A-1	[30,31]
Protein phosphatase 2A-2	AT1G10430	PP2A-2	[30,31]
Protein phosphatase 2A-3	AT2G42500	PP2A-3	[30,31]
Protein phosphatase 2A-4	AT3G58500	PP2A-4	[30,31,33]
Serine/threonine protein phosphatase 2A	AT1G69960	PP2A	[30,31]
Regulatory-associated protein of TOR 1	AT3G08850	RAPTOR1	[31]
2A phosphatase-associated protein of 46 kD	AT5G53000	TAP46	[31]
Target of rapamycin	AT1G50030	TOR	[31]
Protein kinase family protein/WD-40 repeat family protein	AT4G29380	VPS15	[30,31]

(LST8), five subunits of PP2A, the PP2A regulatory subunit TAP46, the serine/threonine protein kinase VPS15, together with a considerable number of ATG proteins (Table 1). Other proteins related to endocytosis and phagophore formation were also shown [31]. In addition, this proteomic analysis led to the identification of more than 1600 proteins with a quantitatively different level of persulfidation under N-deprivation, including key proteins involved in ubiquitin-dependent protein degradation and the ATG3 protein related to autophagy.

Finally, another comparative and quantitative analysis was also performed to detect persulfidated proteins in leaves of Arabidopsis plants exogenously treated with ABA, and curiously, the protein with the highest difference in the level of persulfidation between untreated and ABA-treated samples was the core autophagic protein ATG4a [33]. This investigation was an important breakthrough in determining the specific mechanism of regulation of ATG4 activity by persulfidation, as described below.

ATG4a

The cysteine protease ATG4a is part of the core machinery of autophagy and is involved in the ATG8 conjugation system. Its biological function relies on two different processes during autophagy; first, the cleavage of the C-terminal part of ATG8 in a conserved glycine residue to facilitate its interaction with other ATG proteins, ATG7 and ATG3, and its lipidation of ATG8 by conjugating the protein with a phosphatidylethanolamine (PE) through an amide bond, creating Atg8-PE. The conjugated form, ATG8-PE, is then able to bind to the membranes of the starting phagophore to elongate it and close the autophagosome, but it is also crucial for recognising selective cargoes. ATG4a plays a second function in autophagy, by cleaving the PE from ATG8, which has been referred to as the delipidating process of ATG8, which is necessary for its recycling. Therefore, the function of ATG4a is a prerequisite for autophagy to occur and is one of the most important proteins that regulate autophagosome formation.

The redox regulation of ATG4a has been described in animal systems, Chlamydomonas and *Saccharomyces cerevisiae* [74–77]. In plants, the activity of ATG4a from *Arabidopsis thaliana* was tested *in vitro* and found that ATG4a can also be reversibly inhibited by oxidation using H_2O_2 [75], although further studies must be performed in order to decipher the underlying mechanisms. However, it has recently been reported that overexpression of *Pisum sativum* mitochondrial thioredoxin (PsTRXo1) in tobacco cells (OEX) showed higher gene expression, higher protein content and higher ATG4 activity than control cells. These and further findings suggest the role of TRXs in mediating the redox regulation of autophagy in plant cells [78].

Furthermore, very recently, the regulation of ATG4a by H_2S through the reversible persulfidation of the protein at the Cys170 residue has been described, regulating its proteolytic activity [33]. Using

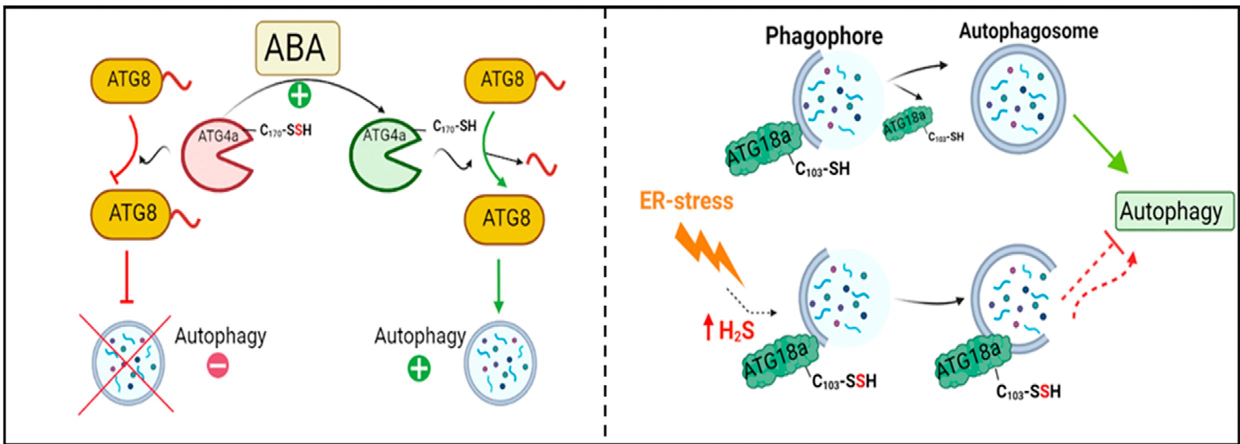

Fig. 2. Schematic regulation of autophagy through persulfidation of specific core autophagy proteins, ATG18a and ATG4a proteins. ABA, abscisic acid; ATG18, autophagy-related gene 18; ATG4, autophagy-related gene 4.

quantitative proteomic analysis, it was found that ABA transiently reduced the persulfidation level of ATG4a. Furthermore, it was shown that ABA induced autophagic flux and that autophagy induction was repressed by H_2S treatment, suggesting that the underlying mechanism of the negative role of sulfide that regulates autophagy was persulfidation of ATG4a, which causes conformational changes in the protein affecting the recognition of the ATG8 substrate [33]. In this way, under basal conditions, the ATG4a is highly persulfidated, and consequently, its proteolytic activity is inhibited, avoided the autophagosome formation. Increased intracellular ABA levels decrease the level of persulfidation of ATG4a activating the ATG8 C-terminal processing and facilitating the progress of autophagy (Fig. 2). The biological significance of this regulation was supported in Arabidopsis leaves under basal and autophagy-activating conditions. A significant increase in proteolytic activity of ATG4 was detected under nitrogen starvation and osmotic stress, which can be inhibited by sulfide.

ATG18a

In a recent publication, another core autophagic protein has been revealed to be a target for persulfidation, ATG18a [34]. This protein is essential for autophagy progression because it forms a complex with ATG2 necessary for autophagosome biogenesis during phagophore expansion [79]. Previous studies demonstrated that transgenic plants with RNA interference-ATG18a had an autophagy-defective phenotype during nutrient stress and senescence [80] and it was necessary to degrade the ER during ER stress by autophagy [81]. Unfortunately, scarce information about the regulation of this protein is found in other systems. The sole references to ATG18 regulation by post-translational modifications have been reported to be phosphorylation in yeast [82] and plants [83]. However, more recently, it was found that ATG18a was regulated by sulfide by persulfidation at cys103 residue (Fig. 2). This persulfidation was reversible by incubation with reducing agents and modification increased the binding affinity of ATG18a to certain phosphoinositides, PtdIns(3)P [34]. In fact, impairment of persulfidation by mutating Cys103 abolished almost completely this binding to phospholipids. The effect of sulfide on its binding capacity was also tested *in vivo*, demonstrating that plants treated with sulfide accumulated ATG18a in the membrane fraction. Furthermore, plants expressing impaired persulfidation ATG18a protein (ATG18a_C103S) showed smaller autophagosomes demonstrating the importance of persulfidation in the correct size of autophagosomes during ER stress. Therefore, it was suggested that reversible persulfidation of ATG18a increases its binding to phosphoinositides and consequently to membranes, but potentially delays its release, inhibiting autophagosome maturation [34]. It is worth noting that this was the first time that sulfide has been involved in the selective degradation of ER by autophagy, providing another level of autophagy regulation in plants, induced by a non-nutritional deficiency stress.

Conclusions and perspectives

Increasing evidence for the signalling role of sulfide in essential cellular processes qualifies persulfidation/S-sulfhydration as a master regulator of plant performance. It is worth noting that the mechanism of

action of sulfide in specific processes has been established. Nevertheless, the number of studies investigating the regulation of autophagy by sulfide is still limited and consequently also the knowledge of this regulatory mechanism. In addition, the correlation among sulfide content increase, the timing thereof, and autophagy activation are also unknown. Another aspect that deserves further investigation in this context is the interplay between sulfide and ROS in the regulation of autophagy.

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Data accessibility

The data that support the findings of this study are available from the corresponding authors (aaroca@us.es; gotor@ibvf.csic.es) upon reasonable request.

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