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THE GLUTATHIONE-DEPENDENT ALARM TRIGGERS SIGNALLING RESPONSES INVOLVED IN PLANT ACCLIMATION TO CADMIUM

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This review focuses on the role of glutathione in the trade-off between antioxidative defence and metal chelation, subsequent signal transduction and acclimation in plant responses to cadmium stress.

Abstract

Cadmium (Cd) uptake from polluted soils inhibits plant growth and disturbs physiological processes, at least partly due to disturbances in the cellular redox environment. Although the sulfur-containing antioxidant glutathione is important in maintaining redox homeostasis, its role as an antioxidant can be overruled by its involvement in Cd chelation as a phytochelatin precursor. Following Cd exposure, plants rapidly invest in phytochelatin production, thereby disturbing the redox environment by transiently depleting glutathione concentrations. Consequently, a network of signalling responses is initiated, in which the phytohormone ethylene is an important player involved in the recovery of glutathione levels. Furthermore, these responses are intricately connected to organellar stress signalling and autophagy and contribute to cell fate determination. In general, this may pave the way for acclimation (e.g. restoration of glutathione levels and organellar homeostasis) and plant tolerance in case of mild stress conditions. This review addresses connections between these players and discusses the possible involvement of the gasotransmitter hydrogen sulfide in plant acclimation to Cd exposure.

Keywords

Autophagy, cadmium, endoplasmic reticulum stress, ethylene, glutathione, hydrogen sulfide, mitochondrial retrograde regulation, phytochelatin, reactive oxygen species, sulfur

1 Glutathione is pivotal in cadmium responses

Cadmium (Cd) is a metal naturally present in soils in low concentrations, although it accumulates above background levels in many areas worldwide. While this accumulation can be reached by natural phenomena, the primary sources of environmental Cd contamination are human activities, including smelting and mining activities, the use of phosphate fertilisers and sewage irrigation in agriculture (Haider *et al.*, 2021; Zulfiqar *et al.*, 2022). Non-essential metals, such as Cd, can even be phytotoxic at low doses (Lin and Aarts, 2012). As Cd is non-biodegradable and has a high bioavailability in the soil, it is taken up by plants and introduced into the food chain, thereby endangering human and environmental health (Clemens *et al.*, 2013).

Cadmium is mainly present in the soil as a divalent cation and can be taken up by transporters designed for essential divalent metals, such as iron (Fe), zinc (Zn) and manganese (Mn) (Zhu *et al.*, 2021; El Rasafi *et al.*, 2022). Once absorbed by plant roots, Cd enters the apoplastic and symplastic pathways to the xylem and phloem, eventually reaching stems, leaves, fruits and seeds (Sterckeman and Thomine, 2020; Zhu *et al.*, 2021). Subsequently, it can disturb physiological processes, for example by affecting electron transport in mitochondria and chloroplasts and inhibit plant growth and development (Haider *et al.*, 2021; Zulfiqar *et al.*, 2022). Such effects often result from a disrupted cellular homeostasis that can be caused by the indirect Cd-induced increase in reactive oxygen species (ROS) (El Rasafi *et al.*, 2022). This occurs shortly after Cd exposure by a depletion in glutathione (GSH) levels in the roots, which results from a fast induction of phytochelatin (PC) biosynthesis (Figure 1) (Jozefczak *et al.*, 2014; Deckers *et al.*, 2020). Glutathione is an antioxidant tripeptide consisting of glutamine, cysteine and glycine, and is synthesised in two steps catalysed by glutamate-cysteine ligase (GSH1) in plastids and GSH synthetase (GSH2) in plastids and the cytosol. Glutathione has multiple roles in maintaining the redox balance. For example, it can reduce hydrogen peroxide (H_2O_2) directly or via participation in the ascorbate (AsA)-GSH cycle (Jozefczak *et al.*, 2012; Hasanuzzaman *et al.*, 2019). Additionally, GSH is the precursor of PCs and is thereby involved in chelating Cd molecules as a consequence of the high affinity of Cd towards thiol groups. Phytochelatins are metal-chelating molecules with a (γ-Glu-Cys)$_n$-Gly (n=2-11) structure synthesised from GSH by phytochelatin synthase (PCS) upon post-translational activation by Cd or other metals (Cobbett, 2000; Jozefczak *et al.*, 2012; Sharma *et al.*, 2016). Once formed within the cytosol, PC-Cd complexes are transported into the vacuole (via two ATP-BINDING CASSETTE (ABCC) transporters, AtABCC1 and AtABCC2, in *Arabidopsis thaliana*) (Park *et al.*, 2012; Sharma *et al.*, 2016). Previous research suggested that the initial GSH depletion, as a consequence of Cd-induced PC production in roots, functions as a redox-dependent alarm triggering different downstream responses (Figure 1) (Deckers *et al.*, 2020).

In addition to altering the redox environment, Cd exposure disrupts the homeostasis of phytohormones such as jasmonic acid, salicylic acid, auxin and ethylene. These phytohormones are involved in plant responses to Cd stress (De Smet *et al.*, 2015; El Rasafi *et al.*, 2022) and are also intertwined (Saini *et al.*, 2021). Nevertheless, this review will focus on ethylene, given its profound changes and signalling role during Cd exposure and its close connection to GSH metabolism, as it is synthesised from the sulfur-containing amino acid methionine (Keunen *et al.*, 2016). Altered ethylene levels and their interplay with the Cd-induced increases in ROS levels may ultimately result in retrograde organellar signalling and autophagy, which can be involved in stress acclimation (Figure 1) (Depaepe *et al.*, 2021). The following parts of this review focus on the downstream signalling processes induced by the GSH-dependent alarm and their contribution to plant signalling and acclimation upon Cd stress, for instance to restore GSH levels, during which hydrogen sulfide (H_2S) potentially plays a fine-tuning role (Figure 1).

2 The role of the cadmium-induced glutathione-dependent alarm in mounting an ethylene response

While crucial during development (Keunen *et al.*, 2016), ethylene is also an important player in the intricate stress response network that allows acclimation or cell death depending on the stress severity that is perceived by the organism (Depaepe *et al.*, 2021). Furthermore, this phytohormone is connected to the antioxidant GSH at multiple levels. For instance, both depend on cysteine for their biosynthesis, although ethylene itself is not a sulfur-containing compound (Keunen *et al.*, 2016). Moreover, in the AsA-GSH cycle, GSH is involved in the reduction of dehydroascorbate to AsA (Zechmann, 2014), which also serves as a co-substrate for the enzyme catalysing the final step of ethylene biosynthesis (Pattyn *et al.*, 2021). Furthermore, Deckers *et al.* (2020) proposed that a transient GSH-dependent change in the redox environment in roots serves as an alarm to elicit ethylene biosynthesis and signalling that contribute to the plant response to Cd (Figure 2). In this context, it was demonstrated that it is the early Cd-induced decrease in root GSH concentrations, rather than an altered GSH redox state, that influences the redox environment, as indicated by an increased H_2O_2/GSH ratio in *A. thaliana* roots during acute and mild Cd stress (Deckers *et al.*, 2020). Cadmium-induced ROS were proposed to be sensed by oxidative signal-inducible 1 (OXI1) (Schellingen *et al.*, 2015b), a protein kinase that is transcriptionally induced and activated by H_2O_2 in *A. thaliana* (Rentel *et al.*, 2004) and is also rapidly and transiently upregulated in *A. thaliana* roots following Cd exposure (Deckers *et al.*, 2020). Its contribution to the activation of mitogen-activated protein kinases (MPK) 3 and 6 (Rentel *et al.*, 2004) may link Cd-induced ROS to increased ethylene biosynthesis (Schellingen *et al.*, 2015b), as these kinases increase 1-aminocyclopropane-1-carboxylic acid (ACC) synthase 2 (ACS2) and ACS6 at the transcript (via the transcription factor WRKY DNA-binding protein 33, WRKY33) and protein levels (Figure 2) (Li *et al.*, 2012). These ACS enzymes catalyse the conversion of S-adenosylmethionine (SAM) to ACC, which is subsequently converted to ethylene by ACC oxidase (ACO) (Keunen *et al.*, 2016; Pattyn *et al.*, 2021). Additionally, it has been demonstrated that ACS2 and ACS6 are important contributors to Cd-induced ethylene emission (Schellingen *et al.*, 2014). Consistent with the proposed MAPK-ACS pathway, a Cd-mediated induction of MPK3 and/or MPK6 activity was reported and appears to be reciprocally linked to ROS (Liu *et al.*, 2010; Jin *et al.*, 2013). Furthermore, both an upregulation of *MPK3* and *MPK6* and an increase in ACC levels were observed in *A. thaliana* roots after only a few hours of Cd exposure. These observations hint at a fast enhancement of ACS protein stability in roots (Deckers *et al.*, 2020). Moreover, the connection between GSH and ethylene is further supported by the observation that simultaneous administration of GSH and Cd at the root level of *Zea mays* seedlings limited the Cd-induced emission of ethylene (Li *et al.*, 2017), while GSH-deficient *cadmium-sensitive 2* (*cad2*) *A. thaliana* plants showed increased root ACC levels (Deckers *et al.*, 2021) and higher ethylene emission (Verena Iven, unpublished results) compared to wild-type (WT) plants after Cd exposure.

Subsequently, a further increase in ACC levels as well as *ACS2* and *ACS6* transcripts after prolonged Cd exposure was hypothesised to indicate a slow transcriptional stimulation of the MAPK-ACS pathway. Notably, it is also at these later stages of the mild stress response that the upregulation of several oxidative stress marker genes and increases in H_2O_2 levels were reported in *A. thaliana* leaves and roots (Deckers *et al.*, 2020). This change in the redox environment may be partly elicited by ethylene (Figure 2), as ethylene biosynthesis *acs2-1 acs6-1* (Schellingen *et al.*, 2015a) and ethylene signalling *ethylene-insensitive 2-1* (*ein2-1*) mutant *A. thaliana* plants generally showed a weaker Cd-mediated induction of several transcripts encoding superoxide ($O_2^{\bullet-}$)-generating respiratory burst oxidase homologues (RBOHs, i.e. NADPH oxidases) and oxidative stress markers in comparison to WT plants (Schellingen *et al.*, 2015b). Such findings in the model organism *A. thaliana* are in line with those in the crop plant *Solanum lycopersicum*, where H_2O_2 levels induced by long-term Cd treatment were lower in leaves of an ethylene-insensitive cultivar, despite their higher Cd concentration (Alves *et al.*, 2017). Furthermore, lower NADPH oxidase and higher superoxide dismutase (SOD) activities as well as lower $O_2^{\bullet -}$ levels were respectively observed in roots and root tips of *A. thaliana* seedlings simultaneously treated with Cd and ACC rather than with Cd alone. The authors hence proposed that ethylene regulates $O_2^{\bullet -}$ levels in the root tip to adjust root architecture during Cd stress (Abozeid *et al.*, 2017). Accordingly, root hair length, ROS, and the induction of the ethylene reporter construct EIN3 binding site (*EBS*)*:GUS* shared a similar pattern across various Cd concentrations in *A. thaliana* seedlings, being stimulated by Cd up to a certain concentration (Bahmani *et al.*, 2022). Taken together, these results suggest that Cd-induced ethylene production, triggered by a GSH-dependent alarm, alters plant stress responses by, for example, influencing the redox environment. Such ethylene-mediated redox changes may subsequently be amplified by further stimulation of ethylene production through the MAPK-ACS pathway, of which the most profound transcriptional induction during Cd stress in leaves indeed generally coincided with H_2O_2 increases (Deckers *et al.*, 2020) and was largely EIN2-dependent (Schellingen *et al.*, 2015b). It should be noted, however, that this MAPK pathway responds to ROS that likely originate from additional sources besides the aforementioned NADPH oxidases, including organelles (Liu and He, 2017; Jalmi *et al.*, 2018).

According to these obtained results and proposed models, the rapid Cd-induced GSH depletion contributes to early ethylene production and signalling that, in turn, closes the loop by influencing the redox environment and stimulating its own production. As this puts forward an initialising role for PC production in stimulating Cd-induced ethylene production, it might seem contradictory that roots of PC-deficient *cad1-3 A. thaliana* also showed stronger *ACS2*, *ACS6* and ethylene response factor 1 (*ERF1*) inductions, ACS activity and

ACC concentrations compared to WT plants following short-term Cd exposure (Chen *et al.*, 2020). This is in accordance with the enhanced ethylene emission found in this mutant compared to WT plants following Cd exposure (Verena Iven, unpublished results). While this mutant might more strongly rely on GSH for Cd chelation, inducing the perception of GSH depletion, it was proposed by Chen *et al.* (2020) that the stimulation of ethylene biosynthesis results from strong Cd toxicity elicited by a lack of PCs. Indeed, preliminary data show that these mutants suffer from a higher extent of oxidative stress following Cd exposure (Verena Iven, unpublished results), which may cause a severe burst of ethylene rather than a controlled increase as a result of a GSH-dependent alarm. This hypothesis further highlights the delicate balance between controlled signalling and damage depending on stress intensity.

3 Restoring glutathione levels during acclimation relies on the cadmium-induced glutathione-dependent alarm and subsequent ethylene signalling

Cadmium exposure in plants stimulates sulfur assimilation (Mendoza-Cózatl *et al.*, 2005; Khan *et al.*, 2016a). The increased expression of sulfate transporter 1;2 (*SULTR1;2*) in Cd-stressed *A. thaliana* was proposed to rely on the depletion of cysteine, the GSH precursor γ-glutamylcysteine (γ-EC) and GSH as well as the shift to a more oxidising environment (Jobe *et al.*, 2012). This suggests that the previously discussed GSH-dependent alarm, as well as subsequent changes in the redox environment, may contribute to Cd-induced sulfur assimilation. Accordingly, prolonged Cd exposure is generally associated with enhanced GSH biosynthesis (Table 1, Figure 1). Nevertheless, caution is advised when comparing the results of studies using different experimental set-ups (e.g. different exposure conditions), as this can affect GSH levels (Koffler *et al.*, 2014).

In contrast to roots showing an initial GSH depletion, leaf GSH levels increased in *A. thaliana* upon mild Cd stress (Jozefczak *et al.*, 2014). As the upregulation of *ERF1* in leaves of Cd-exposed *A. thaliana* preceded the elevation of ACC concentrations in this organ, it was proposed that ACC or ethylene originating from roots following the GSH-dependent alarm during mild Cd exposure activates ethylene signalling in leaves to allow timely induction of GSH biosynthesis (Deckers *et al.*, 2020). This hypothesis is in accordance with the observation that scions of a WT *S. lycopersicum* cultivar contained less sulfur when they were grafted to Cd-exposed rootstocks of an ethylene-insensitive cultivar rather than a WT cultivar and further exposed to Cd for 30 days (Alves *et al.*, 2017). In addition, increases in GSH levels or GSH metabolism-related gene expression after Cd exposure were lower or absent in root tips of *Lycium chinense* treated with the ACC biosynthesis inhibitor 2-aminoethoxyvinylglycine (Guan *et al.*, 2015a) and in leaves of *A. thaliana ethylene response 1-1* (*etr1-1*), *ein2-1* and *ein3-1* ethylene signalling mutants (Schellingen *et al.*, 2015b). Furthermore, it was shown that long-term Cd-induced sulfur assimilation and elevation of cysteine and GSH levels in *Brassica juncea* leaves further increased when plants were additionally treated with ethylene-releasing ethephon (Khan *et al.*, 2016b). Taken together, these results suggest that Cd-induced ethylene production positively affects GSH production (Figure 2).

4 Cadmium-induced organellar responses triggered by the glutathione-dependent alarm and subsequent signalling

Glutathione is present in different subcellular compartments (Zechmann, 2014), where it can be influenced by Cd stress (Kolb *et al.*, 2010; Koffler *et al.*, 2014). In *A. thaliana* mesophyll cells, Cd exposure was found to affect GSH localised in chloroplasts (Koffler *et al.*, 2014), which are also the sites where the first step of GSH biosynthesis predominantly occurs (Zechmann, 2014) and where *de novo* methionine production from cysteine for ethylene biosynthesis takes place (Bittner *et al.*, 2022). Furthermore, chloroplasts are known to be affected by Cd at the level of their photosystems (Parmar *et al.*, 2013). In response to environmental stressors, these organelles can employ various retrograde signalling arms that are activated, among others, via redox signals (Cejudo *et al.*, 2021; Jan *et al.*, 2022). One such example can be the redox regulation of the Calvin-Benson cycle, which is responsible for the production of triose phosphate (Cejudo *et al.*, 2021). This metabolite was reported to activate MPK6 and subsequently induce transcription of ethylene signalling genes following chloroplast-to-cytosol transport via the triose phosphate/phosphate translocator (TPT) in *A. thaliana* as a response to high light stress (Vogel *et al.*, 2014). Accordingly, Cd exposure was shown to alter the chloroplast redox environment (Table 2) and decrease triose phosphate utilisation in hybrid *Pennisetum* leaves (Song *et al.*, 2019). Moreover, it was found to upregulate the expression of *TPT*, which was further enhanced by additional ethephon treatment, in leaves of *Catharanthus roseus* (Chen *et al.*, 2017). While Chen *et al.* (2017) propose that this last observation is linked to the transport of increased catharanthine levels, a potential connection between triose phosphate-dependent retrograde signalling and Cd stress seems plausible and deserves further attention. To the best of our knowledge, it remains elusive whether and how the chloroplast retrograde pathways function during Cd stress. However, it is important to note that chloroplasts play an important role in the Cd response as they are inevitably involved in restoring GSH levels during acclimation.

Furthermore, Cd also causes mitochondrial stress. For instance, clustering has been observed between transcriptional responses to the mitochondrial complex III inhibitor antimycin A and Cd (Yu *et al.*, 2001) and mitochondrial homeostasis pathways were recently reported to represent an important part of the Cd stress response (Liu *et al.*, 2023). In addition, Cd has previously been shown to alter the mitochondrial redox environment (Table 2) (Keunen *et al.*, 2011). To continue electron flow from the tricarboxylic acid (TCA) cycle in mitochondria without excessive ROS production by electron leakage from the electron transport chain (ETC), plants redirect electrons through an alternative pathway relying on alternative oxidase

(AOX) (Keunen *et al.*, 2011). The induction of *AOX1a* is part of a mitochondrial retrograde regulation (MRR) response. Together with other mitochondrial dysfunction stimulon (MDS) genes (e.g. *OXI1*), it can be regulated by the NAC (no apical meristem/*Arabidopsis* transcription activation factor/cup-shaped cotyledon) transcription factor ANAC017 following its cleavage from the endoplasmic reticulum (ER) upon mitochondrial stress (Figure 2) (De Clercq *et al.*, 2013; Ng *et al.*, 2013). It should be noted that ANAC017 was found to also play a role in the retrograde response of chloroplasts upon methyl viologen treatment, indicating that retrograde signalling by ANAC017 can be triggered by both organelles (Van Aken *et al.*, 2016). In addition, the potential involvement of MPK6 and/or phytohormones, including ethylene, in mitochondrial stress signalling has become evident (Wang and Auwerx, 2017; Kacprzak *et al.*, 2020) and a model was put forward where ANAC017 serves as an upstream mediator of ethylene biosynthesis (He *et al.*, 2022). Notably, Cd-induced stress responses share several characteristics with mitochondrial stress signalling. As such, a mitochondrial stress signal stimulating MAPK was observed in Cd-exposed *Oryza sativa*, where inhibition of mitochondrial permeability transition pore opening lowered Cd-induced MPK activity (Yeh *et al.*, 2007). Moreover, Cd-induced *AOX1a* (Keunen *et al.*, 2015) and *OXI1* (Schellingen *et al.*, 2015b) upregulation in *A. thaliana* leaves was generally less pronounced in *acs2-1 acs6-1* and/or *ein2-1* mutants compared to WT plants. These results suggest that Cd stress leads to MRR, which may be (partially) mediated by ethylene that depends on the GSH-dependent alarm (Figure 2).

Apart from the abovementioned organelles, the ER is also an important target of Cd toxicity. Furthermore, it has interesting redox-related properties as it needs to maintain an oxidising environment for oxidative protein folding, which also produces H_2O_2. Interestingly, the ER does not contain glutathione reductase (GR), making the fate of GSSG in this compartment unclear (Meyer *et al.*, 2021; Cao *et al.*, 2022). As such, the ER is an interesting organelle to focus on during GSH-depleting Cd stress. Increased expression of ER stress markers was found in *A. thaliana* seedlings (Xi *et al.*, 2016) and a *Nicotiana tabacum* cell line (Xu *et al.*, 2013) shortly after Cd exposure, suggesting that Cd induces ER stress in plants (Figure 2). This occurs as a reaction to the accumulation of unfolded proteins in the ER, which can be triggered by various stressors and activates the unfolded protein response (UPR). The UPR consists of multiple arms of which the inositol requiring enzyme 1 (IRE1)-dependent splicing of basic leucine zipper 60 (*bZIP60*) mRNA and the proteolytic cleavage of bZIP17 and bZIP28 are the best-characterised in plants. Consequent processing of these bZIPs allows their translocation to the nucleus, where they induce target gene expression to restore proteostasis (Afrin *et al.*, 2020; Cao *et al.*, 2022; Simoni *et al.*, 2022). Although the exact mechanism of UPR induction by Cd requires further characterisation, a role for SAL1 has been uncovered, as a *sal1 A. thaliana* mutant showed less or no upregulation of ER stress response markers compared to WT plants during Cd exposure (Xi *et al.*, 2016). For the generation of sulfate-containing molecules during secondary sulfur metabolism, SAL1 degrades 3′-phosphoadenosine 5′-phosphate (PAP), a by-product resulting from sulfate group donation to target compounds, in chloroplasts and mitochondria (Chan *et al.*, 2019). The activity of this enzyme is repressed by oxidation, allowing PAP accumulation to serve as a chloroplast retrograde signal during oxidative stress (Van Aken and Pogson, 2017; Chan *et al.*, 2019). Consequently, this pathway induces a set of target genes that partly overlaps with that of ANAC017 (Van Aken and Pogson, 2017). This additionally suggests that organellar retrograde signalling is involved in regulating the Cd-induced UPR, which is also in line with the observations that mitochondrial or chloroplast ETC inhibitors influence the expression of several ER stress markers (Ozgur *et al.*, 2015; Fuchs *et al.*, 2022) and that bZIP60 is a target of ANAC017 (He *et al.*, 2022). Furthermore, evidence points towards a reciprocal interaction between ROS and ER stress (Depaepe *et al.*, 2021; Cao *et al.*, 2022) and ethylene is also involved in several ER stress outcomes (Depaepe *et al.*, 2021) and may be linked to UPR induction (Malerba *et al.*, 2010; Czékus *et al.*, 2022). Such connections would hence be an interesting topic for further study of Cd-induced stress responses.

Another outcome of ER stress is autophagy, as recently reviewed (Simoni *et al.*, 2022). During macroautophagy, a double-membranous vesicle (i.e. an autophagosome) surrounds cytoplasmic material and transports it to the vacuole for degradation and subsequent recycling of released nutrients. Although important for growth and developmental processes, it is also induced during stress responses to target damaged material (e.g. proteins and organelles) and generally enhances plant stress tolerance (Signorelli *et al.*, 2019; Su *et al.*, 2020). Autophagic bodies or their remnants have been observed in vacuoles of Cd-treated plants (Figure 4), such as *Glycine max* (Gzyl *et al.*, 2017) and *Triticum aestivum* (Yue *et al.*, 2018), as well as in an *N. tabacum* cell line (Kutik *et al.*, 2014). Moreover, short-term treatment of *A. thaliana* with Cd led to increased transcript and protein levels of autophagy-related 8 (ATG8) (Calero-Muñoz *et al.*, 2019). This ATG protein is involved in autophagosome formation and target isolation and can, due to its presence on autophagosomal membranes following lipidation (Su *et al.*, 2020), serve as an autophagy marker (Klionsky *et al.*, 2021). Although autophagosome formation is a complex process involving a network of other autophagy proteins and post-translational modifications (Su *et al.*, 2020), Calero-Muñoz *et al.* (2019) also observed an autophagosomal membrane containing ATG8 surrounding peroxisomes in leaves of Cd-stressed *A. thaliana*, indicating that Cd exposure triggers selective autophagy of peroxisomes (i.e. pexophagy). While it was suggested that this process may occur due to peroxisomal membrane protein oxidation as a result of Cd-induced ROS, this requires further investigation (Calero-Muñoz *et al.*, 2019). Nevertheless, a reciprocal connection between ROS and autophagy has frequently been observed and reviewed (Signorelli *et al.*, 2019; Su *et al.*, 2020). Hence, it is plausible that a link between redox alterations and autophagy also exists during Cd-induced stress responses. Indeed, autophagy was shown to be involved in controlling peroxisome numbers and might hence influence Cd-induced peroxisomal ROS formation in *A. thaliana* leaves (Calero-Muñoz *et al.*, 2019). Furthermore, a reciprocal connection exists between ethylene and autophagy (Su *et al.*, 2020) and other components of the MAPK-ACS pathway (such as WRKY33 (Lai *et al.*, 2011) and ACC (Zhu *et al.*, 2018)) were found to positively modulate autophagy during stress or enhance ATG8-target interaction (in case of MPK3 (Brillada *et al.*, 2021)). Additionally, other players may connect autophagy and the Cd stress response. Treatment of ER-stressed *Chlamydomonas reinhardtii* with GSH mitigated enhanced ATG8 accumulation. Here, GSH appears to play a specific role, as supplementation with the antioxidative metabolite AsA did not affect the ER stress-induced increased ATG8 abundance. Nevertheless, it remains to be determined whether this occurs through redox changes or via an alternative effect of GSH on the ER (Pérez-Martín *et al.*, 2014) and how this translates to autophagy in plants. Moreover, autophagy induction during drought stress in leaves of ACC-pretreated *S. lycopersicum* was generally stronger in *AOX1a*-overexpressing lines and lower in *AOX1a*-RNA interference lines compared to WT plants. Overall, these effects were less pronounced when ROS levels in these mutants were altered, suggesting that AOX affects autophagy by keeping ROS levels within an appropriate range (Zhu *et al.*, 2018). Accordingly, overexpression of *AOX1a*-regulating *ANAC017* was reported to increase the expression of several ATG genes (Meng *et al.*, 2019). While these results further highlight the delicate balance between (organellar) ROS signalling and autophagy, they again suggest that similar mechanisms may contribute to Cd-induced autophagy and deserve further attention, as ACC/ethylene, GSH and AOX1a are important players in Cd-induced stress responses (Figure 2).

Although ROS, ethylene and the retrograde signalling processes may contribute to plant acclimation to Cd stress, it should be acknowledged that acclimation to stressors depends on additional factors, such as the stress severity (Depaepe *et al.*, 2021). For instance, AOX1a was found to contribute to limiting the upregulation of several oxidative stress marker genes and increasing GSH levels in leaves of *A. thaliana* following exposure to 5 but not 10 µM Cd, suggesting that more severe Cd stress overwhelms its protective function (Keunen *et al.*, 2015). Likewise, the UPR is tied to multiple outcomes with the first goal being the repair of ER function, although this restorative function can switch to cell death following uncontrollable ER stress (Afrin *et al.*, 2020; Simoni *et al.*, 2022). Overexpression of the ER chaperone Binding immunoglobulin Protein (*BiP*) reduced Cd-induced cell death in an *N. tabacum* cell line (Xu *et al.*, 2013) and *G. max* plants (Quadros *et al.*, 2022), and *LcBiP* overexpression in Cd-exposed *N. tabacum* led to higher GSH levels in its leaves (Guan *et al.*, 2015b). Furthermore, there are indications that the UPR, in response to the ER stressor tunicamycin, contributes to raising GSH levels in *A. thaliana* (Ozgur et al., 2014) and *C. reinhardtii* (Yamaoka et al., 2018). Such observations suggest that the UPR contributes to elevating GSH levels and to acclimation. Nevertheless, root growth of the *bzip28 bzip60 A. thaliana* double mutant was less inhibited by Cd treatment compared to that of WT plants (Xi *et al.*, 2016), again indicating that the outcome of the Cd-induced UPR differs depending on the conditions. Furthermore, autophagy may tip the scale towards cell death if the balance between the extent of autophagy and the maintenance of osmotic homeostasis is disrupted (Signorelli *et al.*, 2019). In this process, called mega-autophagy, cell death often occurs as a result of vacuolar hydrolase release into the cytoplasm (Signorelli *et al.*, 2019; Su *et al.*, 2020). Taken together, these results highlight the plasticity of organellar signalling during Cd exposure.

5 Is hydrogen sulfide involved in finetuning acclimation to cadmium stress?

Hydrogen sulfide is a small gasotransmitter with a simple molecular structure, which can easily travel within and between cells. Although it is a signalling molecule, H_2S can become phytotoxic at elevated intracellular concentrations due to restricted cell respiration as a result of the inhibition of mitochondrial respiratory complex IV (Aroca *et al.*, 2021; Aroca and Gotor, 2022). Its production in plants mainly occurs via three processes. First, in the sulfate assimilation pathway, the enzymes sulfite reductase (SiR) in the chloroplast and O-acetylserine-(thiol)lyase (OAS-TL) in the cytosol, chloroplasts and mitochondria produce H_2S (Aroca *et al.*, 2018; Vojtovič *et al.*, 2021). Secondly, β-cyanoalanine synthase C1 (CAS-C1) produces H_2S along with β-cyanoalanine in the mitochondria (Aroca *et al.*, 2018; Liu *et al.*, 2021). Finally, cysteine degradation by cysteine desulfhydrases, the most prominent being L-cysteine desulfhydrase 1 (DES1), is responsible for the cytosolic production of H_2S along with ammonia and pyruvate (Aroca *et al.*, 2018). Recently, the involvement of 3-mercaptopyruvate sulfurtransferases (MSTs) in H_2S production was proposed (Moseler *et al.*, 2021). Nonetheless, their exact roles in this process are just being elucidated in plants and hence require further investigation.

Once H_2S is produced within the cell, it can exert its effects by protein persulfidation. During this post-translational modification, the thiol groups of reactive cysteine residues within target proteins are converted to persulfide (-SSH) groups. Persulfide groups display a stronger nucleophilic character compared to the original thiol groups, rendering the modified cysteines more reactive. However, how H_2S specifically modifies certain targets remains elusive, as the direct thiol-H_2S reaction is thermodynamically unfavourable. Upon persulfidation, proteins display alterations in their enzymatic activities, structures and subcellular localization (Aroca *et al.*, 2018; He *et al.*, 2018). Using the tag-switch method, Aroca *et al.* (2017) identified that at least 5% of the entire proteome is persulfidated in *A. thaliana* leaves under physiological conditions. These persulfidated proteins are involved in a broad range of processes related to, among others, protein synthesis, post-translational modifications and degradation (Aroca *et al.*, 2017).

Upon Cd exposure, the expression of L-cysteine desulfhydrase (*LCD*) and *DES1* is increased in *A. thaliana* roots, resulting in higher H_2S levels (Jia *et al.*, 2016). Additionally, mutants with decreased LCD or D-cysteine desulfhydrase (DCD) activity are more sensitive to Cd stress, whereas overexpressors are less sensitive (Qiao *et al.*, 2015; Zhang *et al.*, 2020). In order to assess the role of H_2S during Cd exposure, exogenous application of the H_2S donor sodium hydrosulfide (NaHS) is frequently used. Supplementing plants with NaHS increased their Cd tolerance as a result of a decreased Cd uptake, reduced Cd translocation from roots to shoots and/or enhanced Cd retention within cell walls, as demonstrated in multiple plant species (Table 3). Additionally, H_2S can mitigate Cd-induced oxidative stress through its positive effects on antioxidant enzyme activities and GSH levels (Table 3), hereby demonstrating that it is additionally involved in regulating the redox environment and related signalling during Cd stress (Figure 2). In conclusion, H_2S appears to contribute to restoring and maintaining GSH levels and restoring the redox environment following the initial Cd-induced GSH depletion resulting from PC synthesis.

Apart from influencing thiol levels, H_2S may additionally influence Cd-induced ethylene production and signalling. For example, postharvest *S. lycopersicum* fruits displayed reduced *ACS* and *ACO* gene expression upon combined NaHS and ethephon application compared to treatment with ethephon alone (Hu *et al.*, 2019). Additionally, NaHS supplementation resulted in reduced ACS and ACO activities, a lower ACC content and eventually reduced ethylene production in postharvest *Prunus persica* fruits (Zhu *et al.*, 2019). This suggests that H_2S inhibits ethylene biosynthesis at the transcript and protein levels. Effects of H_2S on ethylene during vegetative growth were also addressed by exposing *S. lycopersicum* seedlings to NaHS. The results showed that NaHS decreased ethylene concentrations, probably as a consequence of increased SlACO1 and SlACO2 persulfidation and negative regulation of ACO activity (Jia *et al.*, 2018). To the best of our knowledge, no information on the interactions between H_2S and ethylene during Cd stress is available. However, a model was proposed by Sehar *et al.* (2022) in which heat stress induces oxidative stress, consequently increasing sulfur assimilation, which results in increased H_2S and ethylene biosynthesis. The increased H_2S and ethylene concentrations subsequently regulate antioxidants to scavenge heat-induced ROS and thereby induce tolerance. Additionally, H_2S-induced ethylene inhibition and additional crosstalk was suggested to be essential in heat tolerance (Sehar *et al.*, 2022). Furthermore, an interplay between H_2S and ethylene was recently demonstrated to mitigate hexavalent chromium [Cr(VI)] stress in two *Vigna* species. Notably, the authors demonstrated that H_2S acts downstream of ethylene as NaHS supplementation reduced the Cr(VI)-induced increase in $O_2^{\bullet -}$ and H_2O_2 production resulting from inhibited ethylene biosynthesis, whereas ethylene supplementation could not improve effects of inhibited H_2S biosynthesis (Husain *et al.*, 2021). Consequently, it is possible that similar processes are involved in the response to Cd stress. We propose that during Cd exposure, H_2S functions downstream of ethylene to limit ethylene biosynthesis, avoiding long-term persistence of increased ethylene levels which might not be beneficial during acclimation (Figure 1).

Aside from its regulatory effects on GSH and ethylene metabolism, H_2S affects other cellular responses that can be important during plant acclimation to Cd stress, such as mitochondrial homeostasis (Table 3) and autophagy. For instance, NaHS exposure induced the expression of *AOX1a*, *AOX1b* and *AOX1c* in non-stressed *O. sativa* cell suspension cultures (Xiao *et al.*, 2010) and alleviated Cd-induced mitochondrial stress and cell death in *Cucumis sativus* root tips (Luo *et al.*, 2021). Moreover, H_2S was reviewed to affect autophagy through protein persulfidation (Aroca and Gotor, 2022). More specifically, it was shown that 17 *A. thaliana* proteins with a key role in autophagy can be persulfidated (Jurado-Flores *et al.*, 2021). Accordingly, in the *des1-1 A. thaliana* mutant, which has reduced endogenous H_2S levels, autophagy induction appeared to be enhanced compared to WT plants under control conditions (Álvarez *et al.*, 2012). Consequently, it can be concluded that H_2S negatively regulates autophagy (Álvarez *et al.*, 2012; Aroca and Gotor, 2022). Although this might seem contradictory to its potential protective function, we hypothesise that this regulation finetunes autophagy during Cd stress to timely restore autophagic activity to baseline conditions and prevent cell death as a consequence of uncontrolled autophagy.

6 Conclusion and perspectives

As sulfur is a pivotal component of the antioxidant GSH and metal-chelating PCs, it plays a crucial role during the Cd response. As such, an early Cd-induced change in the redox environment is initiated by PC-induced GSH depletion and leads to subsequent signalling events (e.g. related to the phytohormone ethylene). The altered redox environment and ethylene signalling appear interdependent and can also be associated with the induction of organellar stress signalling (e.g. MRR and UPR) and autophagy. Although Cd-induced stress responses have been extensively investigated, these processes are often addressed individually, focusing on one specific cellular compartment, on whole plants or a certain plant organ, as well as on a certain stage of exposure (e.g. the initial defence response or long-term acclimation). As such, their interconnections are often overlooked and require further investigation in multiple organelles and tissues and at different time points following Cd exposure. Furthermore, it is highly relevant to further unravel the role of H_2S in Cd-induced increases in GSH and PC levels. Additionally, we hypothesise that this molecule also finetunes ethylene signalling, mitochondrial homeostasis and autophagy during acclimation in Cd-exposed plants. Identification of persulfidated proteins in Cd-exposed plants will significantly advance our understanding of the involvement of H_2S in plant responses to Cd stress.

Author contributions

All authors contributed to the conceptualization of the review. VI and IV wrote the original manuscript. AC and SH revised the outline and the final manuscript.

Declaration of interests

The authors have no conflicts to declare

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Table 1 Selected studies demonstrating a general increase in glutathione (GSH) biosynthesis upon prolonged exposure of various plant species to cadmium (Cd).

Plant	**[Cd]**	**Exposure duration**	**Parameter**	**Reference**
Arabidopsis thaliana	5 µM $CdSO_4$	1 - 8 d	transcripts GSH biosynthesis genes GSH levels	Hendrix *et al.* (2020)
Brassica juncea	50 µM $CdCl_2$	30 d	GSH levels	Khan *et al.* (2016b)
Hordeum vulgare	5 µM $CdCl_2$	1 - 25 d	GSH levels	Chen *et al.* (2010)
Lycium chinense	100 µM $CdCl_2$	3 - 24 h	transcripts GSH biosynthesis genes GSH levels	Guan *et al.* (2015a)
Oryza sativa	50 µM $CdCl_2$	0 - 7 d	GSH levels	Zhang *et al.* (2013)
Populus x canescens	75 µM $CdCl_2$	27 d	GSH levels	Ding *et al.* (2017)
Triticum aestivum	50 µM $CdCl_2$	10 d	transcripts GSH biosynthesis genes GSH levels	Li *et al.* (2021)

Table 2 Selected studies demonstrating a changed redox environment in mitochondria and/or chloroplasts of various plant species exposed to cadmium (Cd).

Plant	[Cd]	Exposure duration	Response	Reference
Arabidopsis thaliana	50, 100 µM $CdSO_4$	12 - 96 h 7, 14 d	time-dependent changes in mitochondrial and chloroplastic GSH levels	Koffler *et al.* (2014)
	5 mM $CdCl_2$	30 min	↑ mitochondrial roGFP2-oxidation	Schwarzländer *et al.* (2009)
	20 µM $CdCl_2$	1, 2 h	↑ mitochondrial and chloroplastic ROS production in protoplasts	Bi *et al.* (2009)
Cucumis sativus	200 µM $CdCl_2$	48 h	↑ mitochondrial H_2O_2 level	Luo *et al.* (2021)
Hordeum vulgare	30 µM $CdCl_2$	30 min	↑ $O_2^{\bullet -}$ and H_2O_2 production that is affected by mitochondrial complex III inhibition	Zelinová *et al.* (2019)
	10 - 60 µM $CdCl_2$	30 min	↑ $O_2^{\bullet -}$ and H_2O_2 production that is affected by mitochondrial complex I inhibition	Tamás *et al.* (2016)
Nicotiana tabacum bright yellow-2 cells	3 mM $CdCl_2$	1 h	↑ $O_2^{\bullet -}$ production that is affected by mitochondrial complexes I-II and complex III inhibition	Garnier *et al.* (2006)
Nicotiana tabacum	2.24 $mg.kg^{-1}$ $CdCl_2$	10 d	altered transcription and enzymatic activity of redox regulators, including chloroplastic PRXs	Yang *et al.* (2021)
Solanum tuberosum	10, 30 µM $CdCl_2$	5 min (for $O_2^{\bullet -}$) 30 min (for H_2O_2)	↑ $O_2^{\bullet -}$ and H_2O_2 production in isolated mitochondria *in vitro*	Heyno *et al.* (2008)

Table 3 Selected studies demonstrating increased cadmium (Cd) tolerance upon sodium hydrosulfide (NaHS) supplementation as a consequence of increased chelation and antioxidative defence strategies in various plant species.

Plant	**[Cd]**	**[NaHS]**	**Exposure duration**	**Response**	**Reference**
Arabidopsis thaliana	50 µM $CdCl_2$	50 µM	6 h	↑ GSH content	Jia *et al.* (2016)
Brassica juncea	50 µM $CdCl_2$	100 µM	30 d	↑ antioxidant enzyme activity ↑ GSH and AsA content	Kaur *et al.* (2022)
Brassica napus	20 µM $CdCl_2$	50 µM - pretreatment	1 week	↑ Cd retention in cell walls ↓ Cd translocation	Yu *et al.* (2019)
Cynodon dactylon	750 µM $CdCl_2$	500 µM	1 d	↑ antioxidant enzyme activity ↑ GSH content	Shi *et al.* (2014)
Hordeum vulgare	5, 25 µM $CdCl_2$	200 µM	25 d	↓ Cd translocation ↑ antioxidant enzyme activity	Fu *et al.* (2019)
Isatis indigotica	4.5 - 22.5 µM $CdCl_2$	50-200 µM	14 d	↑ Cd retention in cell walls ↓ Cd translocation ↑ PC content ↓ Cd influx	Jia *et al.* (2020)
Populus euphratica cell culture	100 µM $CdCl_2$	50 µM - pretreatment	24-72 h	↑ antioxidant enzyme activity ↑ vacuolar Cd ↓ Cd influx	Sun *et al.* (2013)
Salix matsudana	5 - 30 µM $CdCl_2$	0.3 mM	60 d	↑ antioxidant enzyme activity ↑ Cd retention in cell walls ↑ GSH content	Yang *et al.* (2018)
Trigonella foenum-graecum	1 - 2 mM $CdCl_2$	100 - 200 µM	2 weeks	↑ antioxidant enzyme activity ↑ phenolics and flavonoids	Javad *et al.* (2022)
Zingiber officinale	7.5 mg.l^{-1} $CdCl_2$	0.8 mM	2, 4, 6, 8, 10 d	↑ antioxidant enzyme activity ↑ *PCS* transcription	Chen *et al.* (2022)

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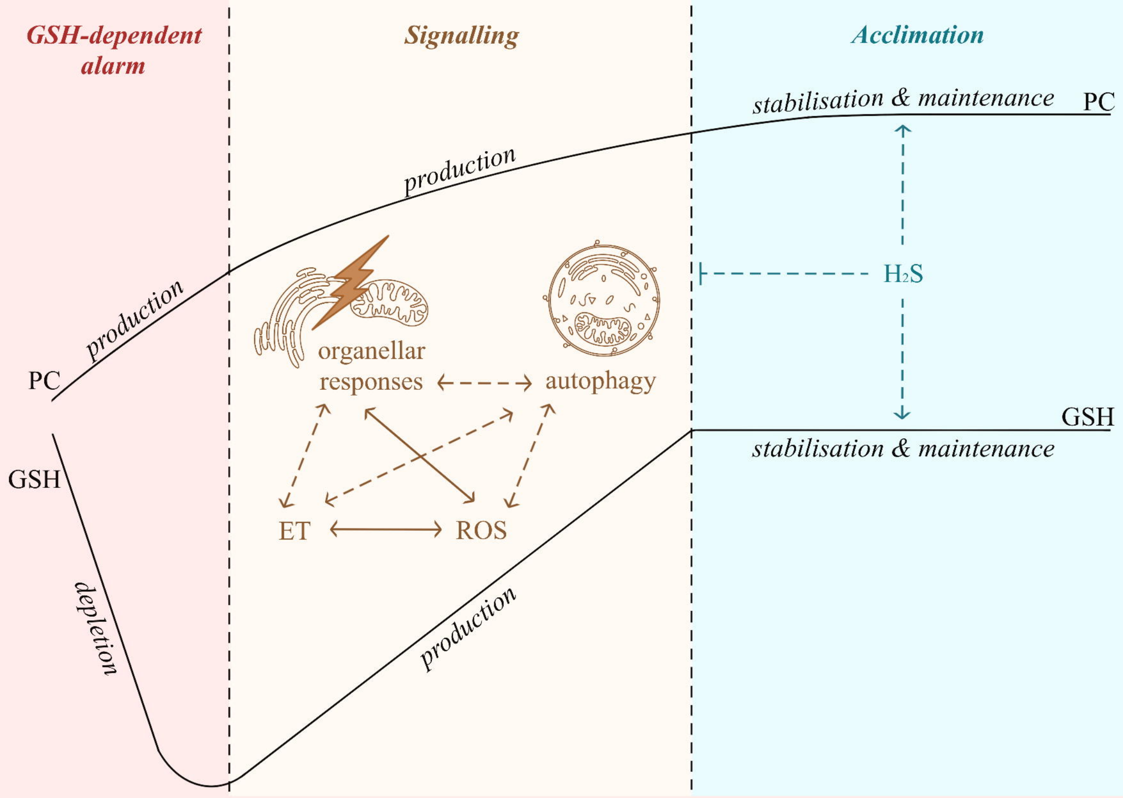
Figure legends

Figure 1: Proposed sequence of events of the mild cadmium (Cd) stress response in plants, showing the glutathione (GSH)-dependent alarm phase, a subsequent signalling phase and an acclimation phase. The GSH-dependent alarm, characterised by a phytochelatin (PC)-induced GSH depletion, triggers ethylene (ET) production and signalling. The subsequent signalling phase is additionally associated with organellar stress responses, elevated reactive oxygen species (ROS) production and autophagy, which are intertwined. Glutathione production is gradually increased to control ROS levels and maintain PC production, while autophagy might be involved in resolving Cd-induced organellar stress. Following prolonged Cd exposure, signalling responses attenuate and both GSH and PC levels stabilise to reach an acclimation state. During this phase, hydrogen sulfide (H2S) may be involved in maintaining GSH and PC production, as well as regulating signalling events. Full lines are based on established interactions, whereas dashed lines indicate interactions that are more speculative.

Figure 2: Hypothetical overview of responses upon cadmium (Cd) exposure in plants. symbols indicate stress responses elicited by Cd, depicting the altered redox environment as well as organellar stress. The (hypothetical) points at which hydrogen sulfide (H2S) acts are indicated using blue plus (positive interaction) or minus (negative interaction) signs. Full lines are based on established interactions, whereas dashed lines are more speculative. ANAC017: no apical meristem/Arabidopsis transcription activation factor/cup-shaped cotyledon 17, ACC: 1-aminocyclopropane-1-carboxylic acid, ACO: ACC oxidase, ACS: ACC synthase, AOX: alternative oxidase, ET: ethylene, GSH: glutathione, MDS: mitochondrial dysfunction stimulon, MPK: mitogen-activated protein kinase, OXI: oxidative signal inducible, PC: phytochelatin, PCS: PC synthase, RBOH: respiratory burst oxidase homologue, ROS: reactive oxygen species, SAM: S-adenosylmethionine, UPR: unfolded protein response.


GSH-dependent alarm
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Acclimation
stabilisation & maintenance
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stabilisation & maintenance
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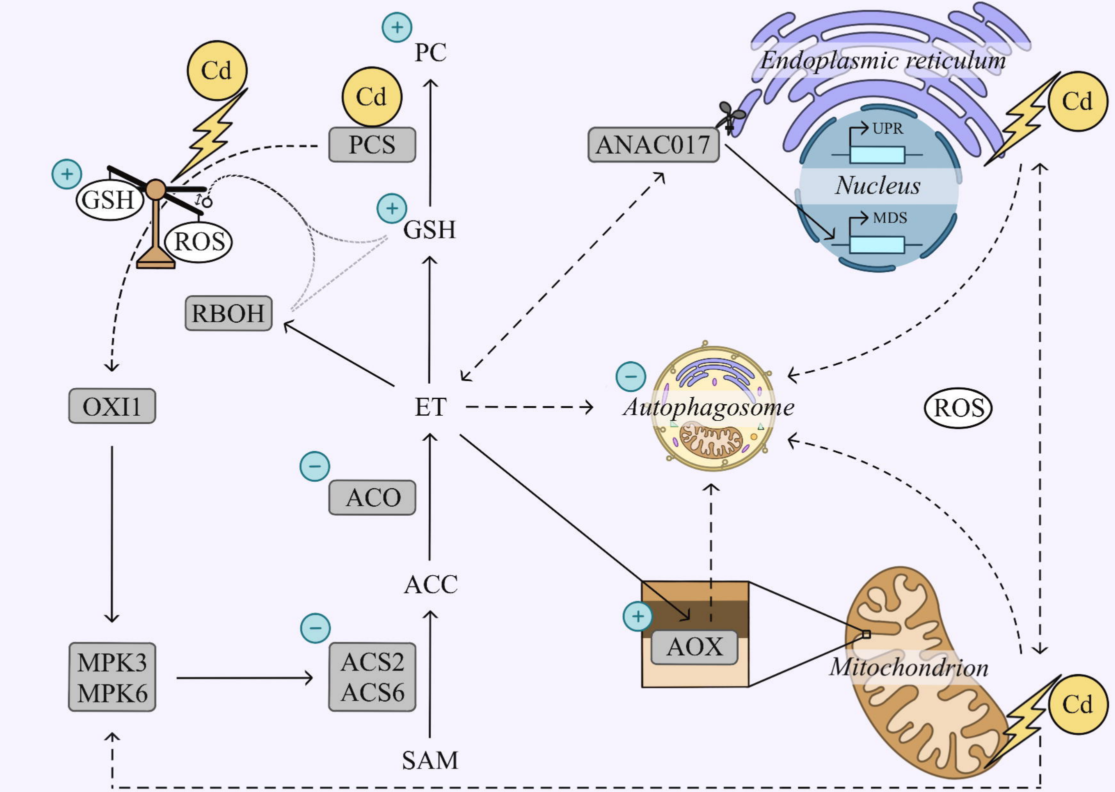
Cd
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ACO
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MPK3
MPK6
−
ACS2
ACS6
SAM
ANAC017
Endoplasmic reticulum
UPR
Nucleus
MDS
Cd
−
Autophagosome
ROS
+
AOX
Mitochondrion
Cd