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Cadmium-induced oxidative stress responses and acclimation in plants require fine-tuning of redox biology at subcellular level

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Abstract

Cadmium (Cd) is one of the most toxic compounds released into our environment and is harmful to human health, urging the need to remediate Cd-polluted soils. To this end, it is important to increase our insight into the molecular mechanisms underlying Cd stress responses in plants, ultimately leading to acclimation, and to develop novel strategies for economic validation of these soils. Albeit its non-redox-active nature, Cd causes a cellular oxidative challenge, which is a crucial determinant in the onset of diverse signalling cascades required for long-term acclimation and survival of Cd-exposed plants. Although it is well known that Cd affects reactive oxygen species (ROS) production and scavenging, the contribution of individual organelles to Cd-induced oxidative stress responses is less well studied. Here, we provide an overview of the current information on Cd-induced organellar responses with special attention to redox biology. We propose that an integration of organellar ROS signals with other signalling pathways is essential to finetune plant acclimation to Cd stress.

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Keywords

Apoplast, cadmium, oxidative stress, reactive oxygen species, signalling, thiols, mitochondria, endoplasmic reticulum, chloroplasts, peroxisomes, nucleus

1. Cadmium exposure rapidly induces oxidative stress

Metals are naturally present in soils, but their levels in the environment are increased as a consequence of polluting anthropogenic activities such as industrial and agricultural applications [1]. They are taken up by plants, thereby entering the food chain, which poses a risk to human health as excess exposure to different metals can induce a variety of diseases [2,3]. Within the pool of metals, essential micronutrients, such as copper (Cu) and zinc (Zn), are cofactors in a multitude of metalloproteins pivotal for normal plant metabolism and are therefore required in minimal concentrations in plants. In contrast, non-essential metals, such as cadmium (Cd) and mercury (Hg), are omnipresent in the environment, but can exert toxic effects on plant metabolism even at low concentrations [3,4]. One general plant response upon exposure to elevated concentrations of both essential and non-essential metals is the occurrence of oxidative stress as a consequence of increased reactive oxygen species (ROS) production and/or decreased antioxidative defence [5]. Depending on their chemical properties, metals elicit oxidative stress via one of two routes. Whereas redox-active metals such as $Cu^{+/2+}$ directly induce ROS production via the Fenton and Haber-Weiss reactions (Figure 1), non-redox-active metals such as Cd^{2+} indirectly cause oxidative stress, e.g. by replacing redox-active metals in metalloproteins or by depleting cellular antioxidants [5,6]. For example, plants exposed to Cd directly stimulate the production of phytochelatins (PCs) via the activation of phytochelatin synthase, thereby depleting cytosolic glutathione (GSH) levels leading to a disturbed cellular redox balance [7,8]. Over the past decades, multiple studies have investigated the imbalance between ROS production and detoxification under Cd stress, ultimately leading to either acclimation and survival or cell death (Table 1).

Whereas Cd-induced plant responses are generally studied at different biological organisation levels, much less is known about the effects of Cd exposure in different subcellular compartments and how these contribute to the overall Cd-induced stress response. Whereas most of the work aimed at unravelling the roles of subcellular ROS production in plant responses to Cd stress has focussed on angiosperms, it should be noted that Cd-induced changes in ROS levels and antioxidant systems have also been reported in other land plants [9–11], as well as in algae [12–16]. Although more detailed studies investigating the effects of Cd at the subcellular level are scarce in these species, it is likely that similar mechanisms to those described in this review are also involved. In this context, *Chlamydomonas reinhardtii* could be particularly useful as a unicellular, photosynthetic model system to further unravel molecular responses to Cd stress at the subcellular level [17].

This review provides an overview of our current knowledge of Cd-induced disturbances in different subcellular compartments and how these lead to ROS production and alterations of the antioxidant pool and redox state to improve the resolution of our view on redox signalling upon Cd exposure.

Furthermore, we will discuss the integration of this oxidative signalling network with other signalling components in fine-tuning plant responses to Cd stress, ultimately leading to acclimation or cell death.

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Table 1: Cadmium-induced ROS production and scavenging in plants. The table catalogues research articles published over the last 5 years (since 2018) that report Cd-induced ROS production and effects on both antioxidant enzymes (transcript levels and/or activity) and metabolites (transcript levels/activity of biosynthetic enzymes and/or concentrations).

Plant Species	Cultivation system	Cd concentration	Exposure duration	AO enzymes		AO metabolites		ROS	Reference
				GE	Activity	GE/Activity	Conc.		
Arabidopsis thaliana	Hydroponics	5 µM $CdSO_4$	24 h-8 d	x		x	X	H_2O_2	[18]
	Solid medium	5-75 µM $CdCl_2$	14 d	x	x	x	X	H_2O_2	[19]
	Hydroponics	50 µM $CdCl_2$	48 h-7 d	x	x	x	X	H_2O_2	[20]
	Solid medium	50-75 µM $CdCl_2$	24 h		x	x	X	H_2O_2, $O_2^{\bullet-}$	[21]
	Solid medium	50-75 µM $CdCl_2$	24 h		x	x	X	H_2O_2, $O_2^{\bullet-}$	[22]
Beta vulgaris	Hydroponics	10 µM $CdSO_4$	2 w		x		X	H_2O_2, $O_2^{\bullet-}$	[23]
Biscutella auriculate	Hydroponics	125 µM $Cd(NO_3)_2$	15 d		x		X	H_2O_2, $O_2^{\bullet-}$	[24]
Brassica campestris/Ipomoea aquatic	Substrate	0.13-17.93 mg Cd/kg	40 d		x		X	H_2O_2, $O_2^{\bullet-}$	[25]
Brassica juncea	Liquid medium	200-600 µM $CdCl_2$	7 d		x		X	H_2O_2	[26]
	Substrate	200 µM $CdSO_4$	31 d		x		X	H_2O_2	[27]
	Hydroponics	80 µM Cd	0-7 d		x		X	H_2O_2, $O_2^{\bullet-}$	[28]
	Substrate	50 µM $CdCl_2$	30 d		x		X	H_2O_2	[29]
Brassica napus	Substrate	250-500 µM $CdCl_2$	7 d		x		X	H_2O_2	[30]
	Hydroponics	10 µM $CdCl_2$	10 d		x		X	H_2O_2, $O_2^{\bullet-}$	[31]
	Hydroponics	10 µM $CdCl_2$	10 d		x		X	H_2O_2, $O_2^{\bullet-}$	[32]
Brassica pekinensis	Hydroponics	50 µM $CdCl_2$	7 d		x		X	H_2O_2	[33]
Glycine max	Substrate	50-300 ppm $CdCl_2$	10 d		x		X	H_2O_2	[34]
Helianthus annuus	Hydroponics	25-100 µM $CdCl_2$	7 d		x		X	H_2O_2	[35]
Hordeum vulgare	Hydroponics	150 µM Cd	48 h		x		X	H_2O_2, $O_2^{\bullet-}$	[36]
Hypogymnia physodes	Liquid medium	10-100 µM Cd	24 h		x		X	H_2O_2	[37]

Isatis cappadocica	Hydroponics / Substrate	100-400 µM $CdCl_2$	5-14 d		x		X	H_2O_2	[38]
Lactuca sativa	Hydroponics	2 mg Cd/L ($CdCl_2$)	4 w		x		X	H_2O_2, $O_2^{\bullet-}$	[39]
Malus sp.	Substrate	50 µM $CdCl_2$	18 d		x		X	H_2O_2, $O_2^{\bullet-}$	[40]
Medicago truncatula	Hydroponics	10-200 µM $CdCl_2$	12 h-12 d	x	x	x	X	H_2O_2	[41]
Mentha arvensis	Substrate	150 mg Cd/kg ($CdCl_2$)	90 d		x		X	H_2O_2, $O_2^{\bullet-}$	[42]
Nicotiana tabacum	Hydroponics	50 µM $CdCl_2$	24-72 h		x	x	X	H_2O_2	[43]
Oryza sativa	Hydroponics	10 µM $CdSO_4$	7 d		x		X	H_2O_2	[44]
	Hydroponics	1-2 mM $CdCl_2$	72 h		x		X	H_2O_2	[45]
	hydroponics	1 mM $CdCl_2$	7-14 d	X			X	$O_2^{\bullet-}$	[46]
	Substrate	100 mg Cd/kg ($CdCl_2$)	Until panicle stage		x		X	H_2O_2	[47]
Panicum maximum	Hydroponics	0.1-0.5 mM $CdCl_2$	9 d	X	x		X	H_2O_2	[48]
Phaseolus lunatus	Substrate	75 mg Cd/kg ($CdCl_2$)	35 d		x		X	H_2O_2	[49]
Phaseolus vulgaris	Liquid medium	50 - 1000 µM $CdCl_2$	6 d		x		X	H_2O_2	[50]
Pisum sativum	Substrate	150 mg Cd/L ($CdSO_4$)	40 d		x		X	H_2O_2	[51]
Populus alba x Populus glandulosa	Substrate	100 mg Cd/kg ($CdCl_2$)	35 d		x		X	H_2O_2, $O_2^{\bullet-}$	[52]
Salix matsudana	Hydroponics	5-30 µM $CdCl_2$	60 d	x	x		X	H_2O_2	[53]
Spinacia oleracea	Substrate	50-100 µM $Cd(NO_3)_2$	40 d		x		X	H_2O_2	[54]
Solanum lycopersicum	Hydroponics	100 µM Cd	7 d		x		X	H_2O_2, $O_2^{\bullet-}$	[55]
	Hydroponics	100 µM $CdCl_2$	10 d	x		x		H_2O_2	[56]
	Substrate	25 µM $CdCl_2$	25 d		x		X	H_2O_2, $O_2^{\bullet-}$	[57]
Triticum aestivum hussa	Hydroponics	100 µM $CdCl_2$	7 d		x		X	H_2O_2, $O_2^{\bullet-}$	[58]
	Hydroponics	50-200 µM $CdCl_2$	90 d		x		X	H_2O_2	[59]
	Substrate	100 µM $CdCl_2$	14 d		x		X	H_2O_2	[60]
	Substrate	15-45 µM $CdCl_2$	3 w		x		X	H_2O_2	[61]

Vigna radiata	Substrate	200 mg Cd/kg ($CdCl_2$)	30 d		x		X	H_2O_2	[62]
	Liquid medium	200 µM $CdCl_2$	10 d		x	x	X	H_2O_2, $O_2^{\bullet-}$	[63]
	Substrate	10-20 mg Cd/kg ($CdCl_2$)	Until podding stage		x		X	H_2O_2	[64]
Withania somnifera	Substrate	5-300 µM $CdSO_4$	45 d	x	x		X	H_2O_2, $O_2^{\bullet-}$,$^{\bullet}OH$	[65]
Zea mays	Hydroponics	100 µM $CdCl_2$	4 d	x	x	x	X	H_2O_2, $O_2^{\bullet-}$	[66]
	Hydroponics	100 µM $CdCl_2$	7 d		x		X	H_2O_2, $O_2^{\bullet-}$	[67]
	Substrate	100 µM $CdCl_2$	14 d		x		X	H_2O_2	[68]
	Hydroponics	85 mg Cd/L ($CdCl_2$)	0 - 4 d		x		X	H_2O_2, $O_2^{\bullet-}$	[69]

2. Cadmium exposure induces ROS production in different subcellular compartments

Soil Cd concentrations and more specifically Cd bioavailability are important determinants of the extent of Cd uptake by plants. The latter depends on multiple soil characteristics, such as pH and organic matter content, as well as on metal speciation. Due to its positive charge, Cd is mainly present as a soil-bound cation or complexed with organic molecules and can enter the roots dissolved in water [70–72]. Although Cd is a non-essential element, it is taken up via transpiration-driven water movement in the apoplast, but for translocation to the xylem and above-ground plant tissues, it has to be transported into the symplast, hijacking transporters for essential metals, such as iron (Fe), manganese (Mn) or Zn [3,71,73,74]. Hence, the presence of these elements in the environment affects plant Cd uptake, as substrate competition for these transporters has been shown [71,72].

Once taken up in plants, Cd indirectly induces oxidative stress by interfering with ROS production and/or detoxification. In addition, it can further enhance oxidative stress levels by triggering signalling pathways affecting these processes. To improve our understanding of Cd-induced disturbances of redox homeostasis, it is important to get more insight into the (sub)cellular localisation of Cd within plant tissues. Whereas Cd distribution studies have been performed in different plant tissues, their resolution is mainly limited to apoplastic and symplastic partitioning in different cell types [3,75] or major subcellular compartments, such as the cell wall and vacuolar fractions [71,76]. In the apoplast, the cell wall forms a protective barrier around plant cells and is a primary target for external threats, such as Cd. High-molecular-weight cellulose microfibrils form the load-bearing structure of the plant cell wall. These fibrils are embedded in a hydrated matrix containing two major types of polysaccharides, hemicelluloses and pectins, and structural proteins contributing to cell wall integrity. Pectins contain a large number of negatively charged galacturonic acid residues that cluster via ionic bridging of non-esterified carboxyl groups by calcium (Ca) ions. Due to these negative charges, the cation binding capacity of the cell wall is significant [77] and is suggested to constitute an important defence strategy to Cd exposure in plants [76,78,79] and algae [80,81]. In the symplast, chelation of Cd by PCs and sequestration into the vacuole is an important defence strategy to restrain Cd intracellularly from metabolically active organelles [76,82,83]. As such, energy-dispersive X-ray microanalysis showed a co-localisation of Cd with sulfur (S) in the symplastic compartments and after transport across the central cylinder of leaves and roots of *Arabidopsis thaliana* seedlings exposed to environmentally relevant Cd concentrations [75]. This points towards Cd chelation by PCs and sequestration into the vacuole on one hand, and interorgan transport of Cd-PC complexes on the other hand [71,84]. Despite the well-studied localisation of Cd in apoplastic and symplastic fractions, little is known about Cd

localisation in different organelles. Recently, Jogawat and colleagues (2021) provided a detailed overview of the presence of different metal transporters in specific organelles and their roles in metal sequestration and redistribution to help plants cope with metal stress [85]. Whereas their review mainly focussed on transporters for essential elements, this information is also highly relevant in the context of Cd entry into these organelles. Nevertheless, more research is needed to unravel the uptake and distribution of Cd into different organelles as well as Cd speciation to shed light on the mechanisms underlying Cd-induced oxidative stress at the subcellular level.

It is well known that Cd induces the production of ROS (Table 1), which can damage DNA, proteins and lipids, but also have the potential to oxidise target molecules such as transcription factors, leading to signalling events [86]. Hydrogen peroxide (H_2O_2) is an uncharged non-radical ROS, which is relatively stable with a half-life in the range of milliseconds [87,88]. As such, it can travel over cellular distances up to 1 µm and act as a primary messenger. Although H_2O_2, occurring in millimolar concentrations in the cell, can act as a signalling molecule in general stress responses, it was proposed that the specificity of ROS produced in distinct subcellular compartments cannot be translated into transcriptional regulation of specific genes required for coping with localised stress at these sites [89]. Nevertheless, it has been shown that H_2O_2 originating specifically from either chloroplasts or peroxisomes has a differential impact on the *A. thaliana* transcriptome. Based on their results, Sewelam et al. (2014) proposed that H_2O_2 originating in specific subcellular compartments triggers two types of responses: one specifically depending on the site of ROS production and one universal response that integrates H_2O_2 signals irrespective of the organelle they originate from [90]. Other ROS such as hydroxyl radicals ($^{\bullet}OH$), superoxide ($O_2^{\bullet-}$) and singlet oxygen ($^1O_2$) have a much shorter half-life compared to H_2O_2, i.e. 1 ns ($^{\bullet}OH$) and 1-4 µs ($^1O_2$ and $O_2^{\bullet-}$), with travel distances ranging from 1 nm ($^{\bullet}OH$) up to 30 nm ($^1O_2$ and $O_2^{\bullet-}$) [87]. Therefore, these ROS react with cellular compounds in the vicinity of their production site, generating oxidised products that can act as second messengers [89]. As such, the presence of S-containing amino acids in proteins at different subcellular locations permits the formation of a wide range of oxidative post-translational modifications (PTMs) that can affect redox-sensitive metabolic components, but can also act as regulatory switches on various signal transduction proteins such as transcription factors [91], which may function as specific subcellular signals [92]. Overall, ROS production is commonly observed upon Cd exposure, but the subsequently activated oxidative signalling pathways may differ depending on the type and quantity of ROS produced as well as the timing and location of ROS production [93]. Therefore, the subsequent parts of this review will zoom in on ROS production in different subcellular compartments, specifically focussing on the sites and importance of ROS production under physiological conditions and during Cd stress (Figure 1).

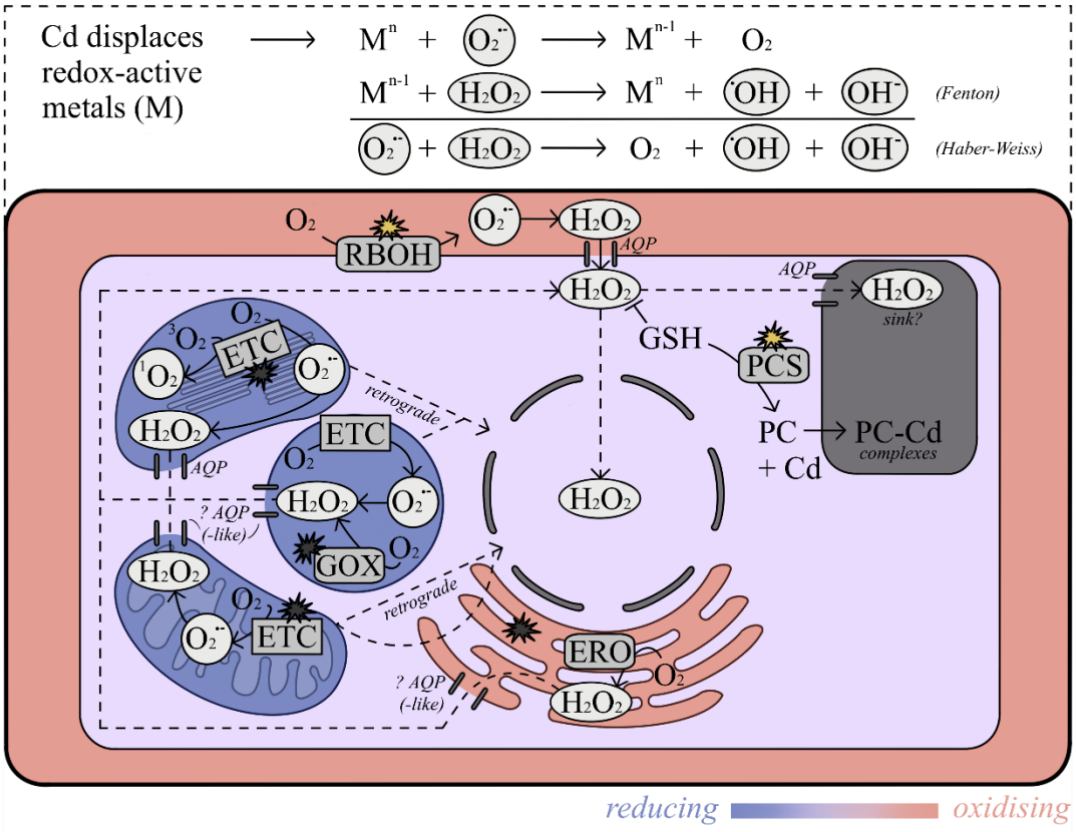

Figure 1: Overview of subcellular ROS production in plant cells under physiological and Cd-exposed conditions. When taken up in plant cells, Cd is immediately complexed with phytochelatins (PC) at the expense of cellular glutathione (GSH). The resulting Cd-PC complexes are sequestered in the vacuole. As Cd is not redox-active, it cannot directly induce ROS production. However, it indirectly enhances ROS levels by disturbing cellular metabolism and electron transport chains (ETC) and displacing essential redox-active metals, such as Fe and Cu. These in turn catalyse Fenton and Haber-Weiss reactions (top figure) leading to the production of highly reactive hydroxyl radicals ($^{\bullet}OH$) in multiple subcellular compartments. The subcellular redox state is highlighted with a colour gradient ranging from red (more oxidising) to blue (more reducing). Whereas the metabolic organelles (chloroplasts, mitochondria, peroxisomes) are the most reducing, the endoplasmic reticulum and apoplast are most oxidising and the cytosol acts as an intermediate buffering zone. Arrows indicate transfer of ROS (mainly hydrogen peroxide; H_2O_2) between subcellular compartments (solid arrows: confirmed; dashed arrows: postulated). Abbreviations: aquaporins (AQP), endoplasmic reticulum oxidoreductin (ERO), electron transport chain (ETC), glycolate oxidase (GOX), hydrogen peroxide (H_2O_2), hydroxide ion (OH^-), hydroxyl radical ($^{\bullet}OH$), superoxide ($O_2^{\bullet-}$), phytochelatin (PC), phytochelatin synthase (PCS), respiratory burst oxidase homolog (RBOH).

2.1 NADPH oxidases producing apoplastic ROS are activated upon Cd exposure

NADPH oxidases (respiratory burst oxidase homologs, RBOHs) are apoplastic ROS-producing enzymes embedded in the plasma membrane that consist of six transmembrane domains, cytosolic C-terminal FAD and NADPH domains and two N-terminal calcium-binding (EF-hand) domains [94,95]. They transfer electrons from cytosolic NADPH, via a transmembrane channel supported by FAD and heme groups, to apoplastic oxygen, producing $O_2^{\bullet-}$ outside of the cell. Activation of NADPH oxidases depends on Ca binding to their EF hand motifs and phosphorylation by Ca-dependent protein kinases [88,96]. Once produced in the apoplast, $O_2^{\bullet-}$ is converted to H_2O_2 spontaneously (favoured by a low apoplastic pH) or potentially via apoplastic superoxide dismutases (SODs) [95,97]. These extracellular ROS can trigger Ca influx into the cell via redox-sensitive Ca channels. Upon entering the cell, Ca can further activate ROS production, thereby creating a spatiotemporal signalling relay [98,99]. In addition, apoplastic ROS can induce oxidative PTMs of cysteine residues, thereby contributing to ROS sensing and signal transduction [91]. Because of the relatively low apoplastic pH (approximately pH 5), oxidation of cysteines is rather limited in this compartment. However, upon environmental stress, a transient alkalization of the apoplast occurs, increasing the reactivity of cysteines with ROS [97]. The diversity of apoplastic ROS together with their downstream activated processes, i.e. Ca release and oxidative PTMs, offers the potential to transmit specific intracellular signals. Furthermore, ROS production in the apoplast also contributes to intercellular communication via the so-called ROS wave, which mediates systemic responses to a variety of stresses and has been extensively described in multiple reviews [88,92,97,99]. Via aquaporins, H_2O_2 can enter the cell and subsequently modify intracellular proteins, thereby functioning as a signal transduction relay to activate downstream cellular responses [100].

Multiple studies have demonstrated that NADPH oxidase activity and *RBOH* gene expression are affected upon Cd exposure and hence are important in regulating Cd-induced stress responses. NADPH oxidase-dependent ROS production was observed in *Cucumis sativus* roots exposed to 10 µM Cd (up to 6 days) [101], BY2 tobacco cells exposed to 3 mM H_2O_2 [102] and root cells of soybean seedlings exposed to 223 µM Cd (up to 24 h) [103]. Although diphenyleneiodonium (DPI), a general flavoprotein inhibitor, is often used to gain insight into the function of NADPH oxidase in different conditions, care should be taken when interpreting the obtained results, as DPI is not a specific NADPH oxidase inhibitor. To further verify the results obtained by DPI treatment, *rboh* knockout mutants can be used. In this regard, differential *RBOH* gene expression was observed in roots and leaves of *A. thaliana* seedlings exposed to 5 µM Cd for 24 h and altered Cd-induced regulation of antioxidant gene expression was observed in *rboh* mutants, further supporting a key role for NADPH oxidases in plant responses to Cd stress [104].

Plants need to continuously respond and adjust to their dynamic, ever-changing environment. To fine-tune their growth and development, they rely on many signals including ROS. As such, NADPH oxidase-derived ROS are involved in a multitude of processes linked to plant development, such as shoot stem cell maintenance, stomatal closure, pollen-stigma interactions, root hair growth as well as defence responses [92,95,96,104]. Cell division and elongation are fundamental cellular processes underlying plant growth. Cell wall loosening is crucial to enable cell elongation and spatiotemporal ROS production plays a major role in this process [95]. The involvement of NADPH oxidases in elongation of specialised cells has clearly been demonstrated for e.g. root hair growth [105–107] and pollen tube formation [108]. In *A. thaliana* seedlings exposed to 40 µM Cd, root hair length was strongly stimulated. This effect was diminished in knockout mutants of *RBOHC*, *RBOHH* and *RBOHJ* and coincided with smaller Cd-induced increases in ROS levels compared to wild-type (WT) plants. However, Cd-induced effects on root hair elongation and ROS levels in *rbohd* and *rbohf* mutants did not differ from those observed in WT plants, indicating that specific RBOH isoforms regulate root hair architecture during Cd stress [109]. In addition, apoplastic H_2O_2 can also be produced by cell wall-anchored class III peroxidases that play an important role in cell wall remodelling. As bicatalytic enzymes, they can oxidise compounds leading to cell wall stiffening, e.g. through lignification, or release ROS, leading to cell wall loosening [110].

Beside cell wall elongation and remodelling, underlying cellular growth and hence plant growth, also physiological regulation by NADPH oxidases through stomatal closure contributes to plant growth and development. Stomatal movements are crucial in the regulation of e.g. photosynthesis and plant water balance through transpiration, but are also responsive to environmental cues [100]. Genetic evidence in Arabidopsis has demonstrated that RBOHD and RBOHF are involved in modulating guard cell ROS production, which is crucial in abscisic acid (ABA)-dependent stomatal closure [111]. As such, altered nutrient translocation was observed in Arabidopsis *rbohd* and *rbohf* knockout mutants exposed to 25 or 100 µM Cd for 1 or 5 days, which was probably due to a disturbed transpiration stream as a result of alterations in stomatal movement [112]. Furthermore, a correlation was observed between *RBOH* gene expression and ABA concentrations in the leaf growth zone of rice plants exposed to 10 or 50 µM Cd [113]. In the same way, production of ROS by NADPH oxidases has also been put forward as a mechanism to reduce Cd uptake. Recently, Hafsi et al. (2022) reported that the extent of Cd uptake was higher in *rbohc*, *rbohd* and *rbohf* mutants compared to WT plants after exposure to 50 µM Cd for 24 h. Furthermore, Cd-induced effects on photosynthesis, stomatal conductance, transpiration and transcript levels of several metal transporters were altered in these mutant backgrounds [114]. In *Brassica campestris* ssp. *chinensis* and *A. thaliana*, the lower extent of Cd uptake after treatment with a nutrient solution aerated with hydrogen gas was associated with enhanced RBOHD-mediated

apoplastic H_2O_2 production. Further analyses in *A. thaliana* revealed that this effect was abolished in the *iron-regulated transporter1 (irt1)* mutant, suggesting that RBOHD regulates Cd uptake via IRT1 [115].

Overall, the activation of NADPH oxidases is an important characteristic of Cd-induced responses (and stress responses in general) to activate defence mechanisms and stress acclimation. Ascorbic acid (AsA) is the major antioxidant in the apoplast, but a system for regeneration of reduced AsA from dehydroascorbic acid (DHA) is lacking in this compartment. Hence, it relies on transport to and regeneration in the cytosol. As a consequence, the redox buffering capacity of the apoplast is relatively low. This means that the apoplast remains in a localised oxidised state to support growth [93,116]. Upon entry into the cell via aquaporins, excess apoplast-derived H_2O_2 can be detoxified by a diverse array of cellular antioxidants to maintain cellular redox homeostasis and hence preserve plant growth and development [100]. The interplay between subcellular spatio-temporal ROS production and downstream signalling molecules as well as the potential cross-talk between the apoplast and intracellular organelles will determine the stress-specific responses underlying plant acclimation. Although research in this field is still in its infancy, unravelling these mechanisms may offer a promising strategy to improve plant Cd tolerance.

2.2 Metabolic organelles produce the majority of ROS in plant cells, which is further stimulated upon Cd exposure

Carbohydrate conversion takes place in chloroplasts (photosynthesis), mitochondria (cellular respiration) and peroxisomes (photorespiration together with chloroplasts and mitochondria). During these processes, ROS are produced as by-products, making these metabolic organelles the major sources of ROS production under physiological conditions. Under light conditions, chloroplasts and peroxisomes are considered the major ROS-producing organelles [93], while in dark conditions and non-photosynthetic organs, the mitochondrial contribution to overall ROS production is much higher [117,118].

Chloroplasts

Photons derived from solar radiation are the driving force to synthesise the energy-rich molecules ATP and NADPH in chloroplasts and are hence an essential part of photosynthesis. To capture photons and subsequently transport highly energetic electrons to the final acceptors, building a proton motive force across the thylakoid membranes, multiprotein complexes, such as photosystem I and II, together with other components of the electron transport chain, are embedded in the thylakoid membrane. Their

function needs to be well coordinated, because malfunctioning of these complexes in the presence of O_2 in the chloroplast can lead to localised elevated ROS production. In photosystem II (PSII), ground state oxygen ($^3O_2$) is excited to singlet oxygen ($^1O_2$) upon its interaction with triplet chlorophyll [119]. Furthermore, $O_2^{\bullet-}$ can be generated in three ways during photosynthesis: (1) through partial oxidation of H_2O at the PSII side, (2) via overreduction of the electron transport chain (ETC) generating plastoquinone radicals and (3) the Mehler reaction at PSI, during which electrons are transferred from ferredoxin to O_2 [120].

Two specific characteristics of chloroplasts make them highly vulnerable to oxidative damage. First, chloroplasts represent the largest Fe sink in most plant cells, containing up to 80% of total Fe in leaves. As many proteins involved in photosynthetic electron transfer require an Fe cofactor, the large Fe pool in chloroplasts is essential for their proper functioning. However, this also entails a risk for oxidative damage through uncontrolled Haber-Weiss reactions [121]. Secondly, the lipid composition of thylakoid membranes mainly consists of the glycerolipids monogalactosyldiacylglycerol and digalactosyldiacylglycerol, which are comprised of high percentages of trienoic fatty acids (i.e. containing three double bonds) such as α-linolenic acid [122]. As a consequence, thylakoid membranes harbour the largest level of lipid unsaturation of any membrane, which renders them particularly vulnerable to lipid peroxidation. It is well known that elevated Cd concentrations interfere with Fe homeostasis [123] and induce lipid peroxidation [113,124]. Therefore, upon exposure to Cd, specific ROS signatures together with oxidised peptides, proteins and metabolites [89,125] can transmit specific chloroplast signals to the cytosol and nucleus to initiate retrograde signalling (cfr. infra, [126,127]).

Interference of Cd with nutrient homeostasis is commonly observed [71], and multiple studies address the effects of Cd on photosynthetic performance [14,123,128–130]. Recently, it was demonstrated that direct Cd-induced inhibition of photosynthesis dominates over the inhibition caused by Cd-induced micronutrient deficiency in the Cd hyperaccumulator *Arabidopsis halleri* in response to sublethal Cd concentrations [131]. Strikingly, inhibition of photosynthetic activities was already observed at very low Cd concentrations of 20 nM in the aquatic species *Ceratophyllum demersum*. Under these conditions, no clear inhibition of respiration was observed, suggesting that the mitochondrial ETC is less susceptible to Cd than the plastidal ETC [132]. The latter is in contrast with findings of another study showing that mitochondrial ROS production precedes ROS production in chloroplasts of *A. thaliana* protoplasts. It should be noted, however, that much higher Cd concentrations were used in these experiments [133]. Also in *Glycine max*, chronic exposure to very low levels of Cd (0.5 nM - 3 µM) led to impaired photosynthesis, probably by inhibiting light-harvesting complex II as well as the further ETC to PSI. Concomitantly, amino acid and carbohydrate metabolism decreased in favour of

antioxidants and detoxifying molecules such as PCs to ensure plant acclimation, but at the expense of plant growth [134]. These results indicate that more research is needed to unravel the specific chloroplastic stress events that lead to ROS production in this subcellular compartment upon Cd exposure. In this context, particular attention should be paid to the experimental design, as Cd concentration, exposure duration and plant species strongly influence the responses observed.

Mitochondria

Whereas plants obtain most of the energy required for cellular processes through photosynthesis, cellular respiration in mitochondria is the main energy-producing process in non-photosynthetic organs and during dark conditions. Here, photosynthetically fixed carbon is oxidised to CO_2 in the tricarboxylic acid (TCA) cycle during which reduced coenzymes are produced. Their electrons funnel into the mitochondrial ETC consisting of complexes I to IV and mobile carriers to finally reduce O_2 and create an electrochemical proton gradient that allows ATP synthesis by ATP synthase (complex V) at the inner mitochondrial membrane. Alternatively, electrons from reduced coenzymes can be transferred to ubiquinone by NAD(P)H dehydrogenases, after which alternative oxidase (AOX) can transfer them to O_2 for its full reduction to H_2O. However, this alternative mechanism does not create a proton gradient [135]. AOX-dependent respiration may be regulated in coordination with other subcellular compartments (e.g. depending on the availability of chloroplast reductants and cytosolic ATP demand) [136] and occurs under both physiological (e.g. seed dehydration) and stress conditions [135]. When mitochondrial electron transport is inhibited, $O_2^{\bullet-}$ is produced at complexes I, II and III [135].

It has been shown that Cd negatively affects the activity of the mitochondrial ETC and increases mitochondrial ROS production. For example, in *Pisum sativum* imbibed with 5 mM Cd for 5 days, a reduced activity of several TCA and respiratory enzymes was observed [137] and *Oryza sativa* plants grown in hydroponics for 10 days with 100 or 200 µM Cd showed decreased activities of complexes I, II and IV [138]. In addition, both *in vitro* and *in vivo* studies highlight that Cd-induced changes in the mitochondrial redox environment may be linked to mitochondrial pulsing [139] and effects on mitochondrial mobility [133]. Enhanced levels of $O_2^{\bullet-}$ were observed in *Nicotiana tabacum* bright yellow-2 (BY-2) cells after exposure to 3 mM Cd, but this effect was less pronounced when cells were pre-treated with inhibitors of mitochondrial complex I and II (rotenone and thenoyltrifluoroacetone) or complex III (antimycin A or myxothiazol) [102]. Isolated mitochondria from *Solanum tuberosum* exposed to 30 µM Cd showed increased levels of $O_2^{\bullet-}$ and H_2O_2, which was also observed after application of mitochondrial complex III inhibitor antimycin A [140]. In addition, 1 h after exposure of *A. thaliana* protoplasts to 20 µM Cd, co-localisation of ROS staining with mitochondria was observed

before co-localisation with chloroplasts, suggesting that Cd-induced mitochondrial ROS production precedes that in chloroplasts [133]. It was reported that Cd exposure induces the oxidation of mitochondrially targeted roGFP sensors in *A. thaliana* seedlings [141] and root epidermal cells [139], suggesting that Cd exposure enhances mitochondrial ROS production. Also in young plants, increases in H_2O_2 levels were observed in mitochondria. This was for example the case in leaves of 28-day-old *P. sativum* exposed to 50 µM Cd for 14 days [142], in roots of *C. sativus* plants exposed to 200 µM Cd for 48 h [143] and in roots of *Hordeum vulgare* after 1 and 3 h exposure to a range of Cd concentrations up to 50 µM. As Cd-induced increases in H_2O_2 levels were lower when plants were co-treated with the mitochondrial complex I inhibitor rotenone, the authors postulated that Cd-induced mitochondrial ROS production is limited by decreasing the electron flow to mitochondrial complex III [144]. Consistently, $O_2^{\bullet-}$ and H_2O_2 levels in *H. vulgare* were altered rapidly after application (30 min) of 30 µM Cd when the mitochondrial complex III inhibitors myxothiazol and antimycin A were supplied, further suggesting that complex III plays a key role in Cd-induced mitochondrial redox changes [145].

Mitochondrial ROS can be associated with Cd-induced cell death as this process was inhibited by addition of mitochondrial ETC inhibitors to Cd-exposed BY-2 cells [102] or *H. vulgare* roots [144]. Indeed, cell death was previously shown to be accompanied by the opening of the mitochondrial permeability transition pore, as well as cytochrome c and/or Ca release from mitochondria in root tips of *C. sativus* seedlings exposed to 200 µM Cd for 48 h [143,146]. To avoid Cd-induced mitochondrial damage, ROS production can be limited through the activation of the alternative respiration pathway. As such, acute exposure to 5 or 10 µM Cd was found to stimulate the expression of *AOX*, *NDA*, *NDB* and/or *UCP* genes, depending on the investigated tissue, in *A. thaliana* leaves and roots [147]. Consistently, AOX protein abundance can also be increased upon Cd treatment in various plants species, as observed for *A. thaliana* [147], *H. vulgare* [148] and *Zea mays* [149], indicating that alternative respiration is stimulated under Cd stress. This may contribute to Cd tolerance in plants, as cell viability of leaves and roots of *Solanum lycopersicum* plants exposed to 250 µM Cd decreased when they were additionally treated with the AOX inhibitor salicylhydroxamic acid (SHAM) [150]. In addition, increases in malondialdehyde content (i.e. a marker for lipid peroxidation) and electrolyte leakage in roots of *H. vulgare* treated with 150 µM Cd for 48 h were higher when additionally supplemented with SHAM [36,151]. Interestingly, Cd-induced increases in alternative respiration and AOX1 transcript and protein levels were also observed in the green alga *C. reinhardtii* [152], suggesting that this strategy is conserved among photosynthetic organisms.

Peroxisomes

Peroxisomes are involved in photorespiration together with chloroplasts and mitochondria, but also perform a diversity of additional functions [118,153]. Oxygenation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in the chloroplasts initiates the inter-organellar photorespiration pathway. In photosynthetic tissues under photorespiratory conditions, the order of magnitude of estimated subcellular H_2O_2 production rates goes from peroxisomes (+/- 10000 nmol m^{-2} s^{-1}) over chloroplasts (+/- 4030 nmol m^{-2} s^{-1}) to mitochondria (< 216 nmol m^{-2} s^{-1}) [118,120,154]. The majority of peroxisomal H_2O_2 (up to 70%) is produced during the first step of the photorespiratory pathway in which glycolate is oxidised to glyoxylate by glycolate oxidase (GOX), but also other enzymes such as acyl-CoA oxidase, urate oxidase, polyamine oxidase, Cu amine oxidase, sulfite oxidase and sarcosine oxidase contribute to peroxisomal H_2O_2 production [118,155]. Furthermore, the NADH/NADPH-dependent ETC in the peroxisomal membrane constitutes a source of $O_2^{\bullet-}$ production in peroxisomes [118,156]. Photorespiration-derived H_2O_2 production is further stimulated by Cd exposure as observed in leaves of *P. sativum* plants exposed to 50 µM Cd for 14 days [142], and 10-day-old *A. thaliana* seedlings exposed to 100 µM Cd during 24 h [157]. Analyses of H_2O_2 dynamics in peroxisomes using the HyPer biosensor revealed that not all peroxisomes have similar H_2O_2 accumulation rates and that a H_2O_2 gradient exists inside these organelles [157]. This was also observed in Cd-treated pea plants using $CeCl_3$ chemistry [142]. The presence of H_2O_2 gradients inside peroxisomes suggests a functional distribution of H_2O_2-producing peroxisomal proteins and antioxidants, but further research is needed to determine the underlying mechanism [157]. Peroxisomes can regulate their number, morphology, movement and metabolism to changes in their environment due to their high plasticity [118,158,159]. A higher number of peroxisomes was also observed in *A. thaliana* seedlings exposed to 60 µM Cd for 12 days [160] or acutely exposed (0.5 - 3 h) to 100 µM Cd [161]. As peroxisomes harbour the majority of ROS produced during environmental stresses, excessive numbers of peroxisomes can produce severe disturbances in redox homeostasis and hence it is essential to tightly control peroxisome populations [162]. Macroautophagy has been demonstrated to play an important role in controlling peroxisome dynamics in *A. thaliana* seedlings under Cd conditions. A transient stimulation of pexophagy at the transcriptional and protein level to degrade excess peroxisomes was observed in *A. thaliana* seedlings exposed to 100 µM Cd for 24 h [157]. This coincides with a previously reported transient peroxisome proliferation in Cd-exposed Arabidopsis leaves [158]. The initiation of Cd-induced pexophagy was accompanied by a rise in peroxisomal H_2O_2 levels and a time-dependent increase in total oxidised protein levels [157]. The authors postulated that oxidation of a specific peroxisomal protein induced by enhanced peroxisomal ROS production might form the trigger for Cd-induced pexophagy. Increased GOX activity could enhance peroxisomal H_2O_2 production to initiate pexophagy, as *gox* mutants displayed an attenuated decrease in the abundance of the peroxisomal marker protein PEX14 between

6 and 9 h after Cd exposure, possibly pointing towards a decreased extent of pexophagy. The same was observed in an *rbohc* mutant, indicating that there might be communication between apoplastic and peroxisomal compartments, which needs further investigation. Overall, the authors suggest that pexophagy is essential in rapid cell responses to Cd stress by regulating peroxisomal populations and their quality in order to avoid disturbances in the cellular redox balance [157].

2.3 Does Cd-induced endoplasmic reticulum stress contribute to disturbances of cellular redox homeostasis?

In contrast to chloroplasts, mitochondria and peroxisomes, which are responsible for cellular carbon metabolism, the endoplasmic reticulum (ER) is mainly involved in lipid and protein biosynthesis and is a major cellular Ca store. Furthermore, this central network of interconnected membranes also synthesises and accommodates many hormone receptors such as those involved in ethylene signalling. At least one third of all newly synthesised cellular proteins enter the ER for glycosylation, folding and/or assembly into protein complexes [163,164]. For example, many proteins rely on intramolecular disulfide bond formation for their structure and proper functioning. Such post-translational protein modifications can be introduced within the ER and other oxidising subcellular compartments through a process known as oxidative protein folding. In the ER, the formation of intramolecular disulfide bonds is primarily mediated by the concerted action of protein disulfide isomerases (PDIs) and endoplasmic reticulum oxidoreductins (EROs) [165]. First, cysteine oxidation and subsequent disulfide formation within substrate proteins is catalysed by oxidised PDIs. Subsequently, the reduced PDIs are re-oxidised by EROs via disulfide exchange. In the final step of the process, electrons are transferred from EROs to O_2, thereby generating H_2O_2 in the ER lumen [166]. Due to a lack of glutathione reductase (GR), the ratio of glutathione disulfide (GSSG) to reduced GSH in the ER lumen is higher than that in other subcellular compartments, ensuring the stability of the intramolecular disulfide bonds formed in substrate proteins [167]. Whether H_2O_2 produced by electron transfer to O_2 leaves the ER for detoxification in the cytosol or remains in the ER lumen, thereby further contributing to the oxidising environment required for oxidative protein folding, remains elusive [166].

In response to a variety of stress conditions, the protein folding capacity of the ER is overwhelmed, resulting in the accumulation of unfolded or misfolded proteins. This situation is referred to as ER stress and activates the unfolded protein response (UPR), which serves to restore cellular protein homeostasis by transcriptionally inducing the expression of genes involved in protein folding or ER-associated degradation (ERAD) or activating autophagy. In plant cells, the UPR consists of two branches, which are regulated by inositol-requiring enzyme 1 (IRE1) and basic leucine zipper 28 (bZIP28),

respectively. Upon accumulation of unfolded or misfolded proteins, IRE1 is activated and catalyses unconventional splicing of the *bZIP60* mRNA, inducing a frameshift mutation that removes the ER anchor. The resulting bZIP60 protein translocates to the nucleus where it enhances the expression of ER stress-responsive genes such as those involved in oxidative protein folding. In the second UPR branch, binding of the ER chaperone binding protein (BiP) to unfolded proteins causes its dissociation from bZIP28, which subsequently translocates to the Golgi where proteases release its transcription factor domain into the cytosol. Similar to bZIP60, bZIP28 can then move to the nucleus to induce the expression of its target genes [168,169].

Several recent studies have shown that Cd exposure induces ER stress and UPR activation in plants. Indeed, Xu et al. (2013) reported increased transcript levels of two ER stress-related genes and the bZIP60 transcription factor in *N. tabacum* BY-2 cells in response to Cd stress. Their results suggest that ER stress negatively impacts cell survival, as treatment with the chemical ER chaperones 4-phenylbutyric acid (4-PBA) and tauroursodeoxycholic acid (TUDCA) relieved Cd-induced programmed cell death (PCD). Furthermore, Cd-induced ER stress and PCD in BY-2 cells were mitigated by heterologous expression of the *A. thaliana BiP2* ER chaperone [170]. In *A. thaliana*, Cd exposure triggered unconventional *bZIP60* splicing and enhanced expression of several ER stress-responsive genes. Plant sensitivity to Cd was significantly reduced in a *bzip28 bzip60* double mutant and wild-type plants treated with 4-PBA and TUDCA [171]. Evidence for Cd-induced ER stress was also provided by transcriptional upregulations of several UPR genes in leaves of Cd-exposed *G. max*. This effect was mitigated in transgenic lines overexpressing *BiP-2* or *BiP-4*, which also showed an increased Cd tolerance at the phenotypic level. Interestingly, these lines also showed lower H_2O_2 levels in comparison to WT plants upon Cd exposure, hinting towards a connection between Cd-induced ER stress and disturbances of redox homeostasis [172]. Such connection was also suggested by Guan et al. (2015), who reported that tobacco plants overexpressing *BiP* displayed enhanced Cd tolerance, concomitant with lower ROS levels, a higher GSH content and decreased antioxidative enzyme activities in response to Cd exposure compared to non-transgenic plants. The authors proposed that BiP relieves endogenous oxidative stress by promoting ROS scavenging. Nevertheless, the molecular mechanisms mediating this response require further investigation [173].

Although information on the interplay between ER stress and redox homeostasis upon Cd exposure is scarce, studies investigating plant responses to tunicamycin (Tm; a compound inducing ER stress by blocking *N*-linked glycosylation) could provide insight into their connection. Within 10 minutes after Tm exposure, *RBOHD* and *RBOHF* expression were significantly enhanced in *A. thaliana*, pointing towards an important role for NADPH oxidases in ER stress-induced ROS signalling. This was further

supported by the fact that suppression of NADPH oxidase activity by DPI alleviated Tm-induced increases in H_2O_2 levels [174]. Activation of NADPH oxidases in response to Tm is likely mediated by ER stress-induced Ca release from the ER into the cytosol. Furthermore, ER stress might alter the cellular redox state by enhanced ERO activity and consequent H_2O_2 production following UPR activation. Increased ROS levels can subsequently influence antioxidative enzyme activities and GSH metabolism [175]. Interestingly, treatment with compounds increasing ROS concentrations in specific subcellular compartments (rotenone for mitochondria, methyl viologen and DCMU for chloroplasts and 3-amino-triazole for peroxisomes) caused discrete transcriptional signatures of ER stress- and UPR-related genes. Furthermore, lower ROS concentrations seemed more effective in triggering ER stress than higher ROS concentrations. These findings indicate that ER stress responses depend on the subcellular source of ROS as well as their concentration [176]. For an extensive review on the UPR-ROS interplay in plants, readers are referred to Ozgur et al. (2018) [175] and Depaepe et al. (2021) [169].

Whether Cd-induced ER stress in plant cells is a direct consequence of Cd entry into the ER or is indirectly caused by metabolic effects of Cd is currently unknown. However, results of a study in yeast suggest that Cd enters the ER. A yeast strain expressing the Cd^{2+} P-type ATPase CadA from *Listeria monocytogenes* in the ER membrane likely accumulates enhanced Cd concentrations in the ER and is hypersensitive to low Cd concentrations. Furthermore, the UPR is activated in this strain at Cd concentrations as low as 1 µM, whereas this is not observed in the control strain [177]. Together, these data suggest that Cd-induced ER stress in yeast is directly linked to Cd accumulation in the ER. Whether the same holds true for plant cells remains to be investigated. Nevertheless, ultrastructural alterations of the ER in response to Cd stress observed in cells of different plant species support this hypothesis [178–181].

2.4. H_2O_2 diffusion from subcellular compartments to the cytosol - an integration hub for ROS under Cd exposure?

Based on its characteristics, it is clear that H_2O_2 is the most stable among the different ROS and it is therefore suggested to travel between different subcellular compartments and adjacent cells, being part of the intra- and intercellular communication system [88,92]. Upon Cd exposure, H_2O_2 is elevated in different subcellular compartments and could subsequently diffuse into the cytosol, which might act as a buffering zone due to the presence of a wide variety of antioxidative mechanisms [93]. Since H_2O_2 cannot readily cross cellular membranes, its transport between subcellular compartments depends on aquaporin-like structures [88]. The regulation of aquaporins has been well studied in the context of apoplast-derived H_2O_2 [100]. Furthermore, aquaporins are likely found in the chloroplast inner

envelope [182]. A similar mechanism for H_2O_2 diffusion from metabolic organelles and the ER is postulated, but the presence of aquaporins in these compartments and their function need to be further investigated [88]. Removal of an overload of cytosolic H_2O_2 into the vacuole can be facilitated by tonoplast aquaporins [88] and this vacuolar sink for H_2O_2 is speculated to function in detoxification processes [117]. In response to Cd exposure, PC production is directly stimulated to detoxify Cd ions at the expense of cytosolic GSH levels [7,8,183]. As such, the H_2O_2/GSH ratio rapidly increases in Arabidopsis seedlings acutely exposed to 5 µM Cd [8]. Under these conditions, the vacuolar detoxification system might operate in concert with the cytosolic antioxidant machinery to maintain cellular redox homeostasis. To further unravel H_2O_2 dynamics in Cd-exposed plants at subcellular resolution, genetically encoded biosensors, such as roGFP2-Orp1 and HyPer7, targeted to different subcellular compartments are powerful tools [184,185].

The origin of ROS in the nucleus is still under debate, as no nuclear aquaporins have been described in plants. Nevertheless, H_2O_2 could diffuse into the nucleus either from the cytosol via the nuclear pores or directly from other organelles via liquid tubules such as stromules (from plastids), matrixules (from mitochondria) and peroxules (from peroxisomes). However, this topic needs further investigation [93]. Increased ROS levels in the nucleus can cause DNA damage and hence cell cycle arrest [186] and it has been reported that a more oxidised nuclear redox state in GSH-deficient Arabidopsis mutants was accompanied by cell cycle arrest [187]. To cope with DNA damage, plant cells rely on the DNA damage response (DDR), which induces cell cycle arrest to allow for DNA repair. Alternatively, the DDR can induce PCD. The master regulator of this pathway in plants is the transcription factor SUPPRESSOR OF GAMMA RESPONSE 1 (SOG1), which is part of the NAM, ATAF1/2, and CUC2 (NAC) family [18]. In response to DNA double-strand breaks (DSBs), SOG1 is activated via phosphorylation of consensus SQ amino acid motifs by ataxia-telangiectasia mutated (ATM). It is also a phosphorylation target of ATM- and RAD3-related (ATR), which responds to DNA single-strand breaks (SSBs) and replication fork defects [188]. In response to DNA-damaging conditions, SOG1 regulates the expression of a plethora of genes involved in DNA repair, cell cycle regulation and PCD. In addition, it also induces the expression of many other transcription factors and target genes, such as OXIDATIVE SIGNAL INDUCIBLE 1 (OXI1) [189], a serine/threonine protein kinase that displays increased gene expression and protein activity in response to a wide range of H_2O_2-generating stimuli [190,191]. Exposure of *A. thaliana* seedlings to 5 µM Cd resulted in the inhibition of cell cycle progression concomitant with the transcriptional upregulation of DDR genes as well as oxidative stress hallmark genes [192]. Interestingly, *OXI1* gene expression was also shown to be induced upon Cd exposure [18,193]. Functional analysis of the *sog1-7* knockout mutant revealed that the upregulation of these genes depends on SOG1. In addition, other

Cd-induced responses related to the oxidative challenge were also disturbed in the *sog1-7* mutant, as indicated by delayed Cd-induced increases of H_2O_2 and GSH concentrations. Whereas a role in oxidative signalling can be attributed to SOG1, it remains to be understood whether redox-based mechanisms play a role in the modulation of SOG1 activity and by extension in DNA damage sensing, signalling, and repair [186].

3. Integration of the redox balance in different subcellular compartments with other signalling pathways mediates Cd-induced plant responses essential for acclimation

ROS-dependent signalling largely depends on the ROS chemistry, their production site and the cellular perception and processing mechanisms determining their specificity [89,117]. Whereas ROS production in metabolic organelles during stress conditions results from metabolic perturbations, NADPH oxidase-dependent ROS production is a spatio-temporally controlled mechanism responding to developmental and environmental cues. Such subcellular ROS production transfers specific signals to the cytosol, which might consist of ROS themselves as well as their oxidation products, but more research is needed to fully understand redox signal transmission to the cytosol [125]. The amount of ROS and hence their downstream oxidised products is determined by the presence of ROS detoxification systems that consist of antioxidant enzymes and metabolites. The latter comprise redox couples that mainly rely on NAD(P)H as the final electron donor to fuel the regeneration of their reduced forms.

3.1. The redox state in different subcellular compartments determines their function and signalling

Different subcellular compartments are characterised by different oxidation states (Figure 1). Whereas the ER is the most oxidising, the metabolic organelles are more reducing and the cytosol can act as an intermediate buffering zone [88]. Whereas no information is available regarding the redox state of the nucleus, we postulate that it is close to that in the cytosol because of the close interaction and ROS diffusion between both compartments. Moreover, nuclear pores are assumed to allow unrestricted bidirectional diffusion of GSH across the nuclear envelope [194] and no differences between both compartments were observed when using genetically encoded biosensors to measure the GSH redox potential [195,196].

The ER and apoplast, which are both more oxidising compartments, are characterised by a limited amount of antioxidants (Figure 2). Ascorbic acid is the main antioxidant in the apoplast, but as it cannot be regenerated in this compartment, transport of DHA to the cytosol for regeneration is crucial [197]. Although the presence of extracellular SODs has been suggested, the majority of NADPH oxidase-

generated extracellular $O_2^{\bullet-}$ is spontaneously reduced to H_2O_2. Both ROS are important in cell wall loosening. Furthermore, H_2O_2 serves as an apoplastic oxidative signal that can be sensed by receptor-like kinases [198] or transported into the cytosol where it interacts with intracellular signal transduction pathways to regulate downstream cellular responses upon environmental stresses.

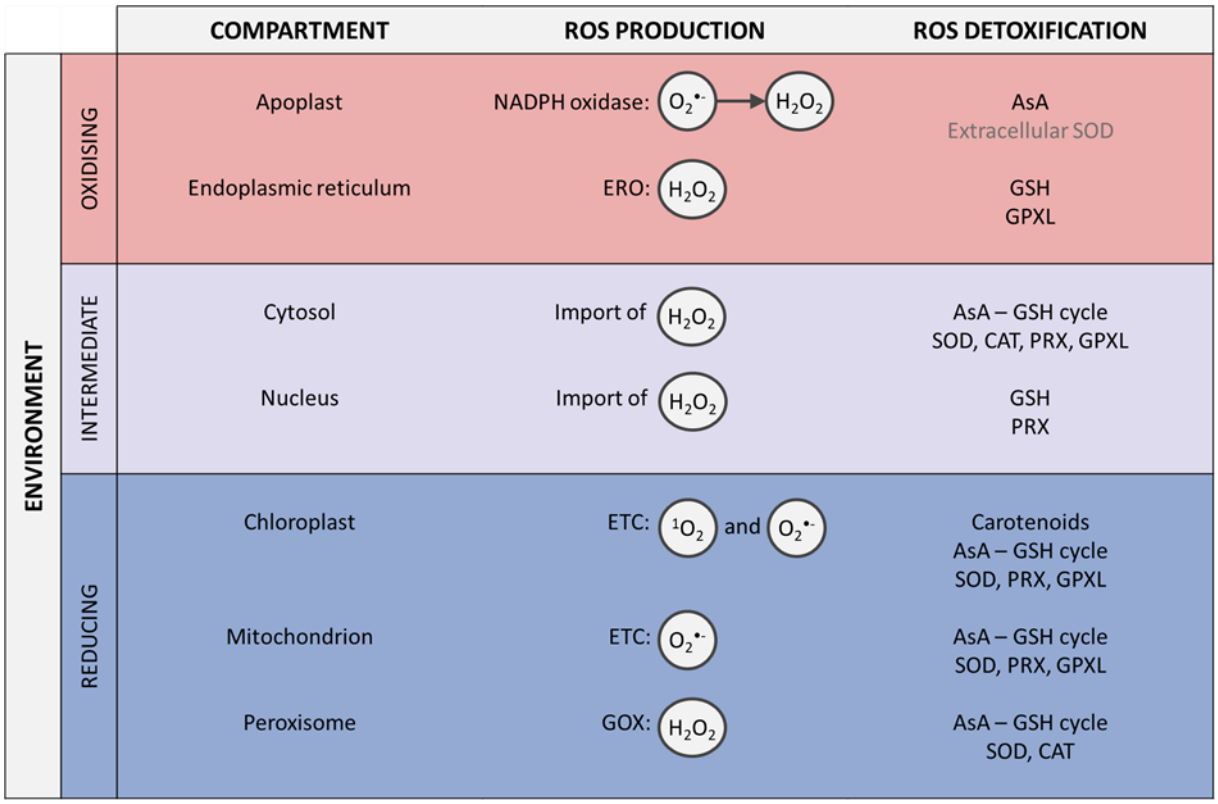

Figure 2: Major subcellular ROS production and detoxification mechanisms in plants. Compartments are categorised based on their oxidation state. Abbreviations: hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), superoxide ($O_2^{\bullet-}$), ascorbic acid (AsA), catalase (CAT), endoplasmic reticulum oxidoreductin (ERO), electron transport chain (ETC), glycolate oxidase (GOX), glutathione (GSH), glutathione peroxidase-like (GPXL), peroxiredoxin (PRX), superoxide dismutase (SOD).

Similar to the apoplast, the ER has very limited antioxidative defence mechanisms. Although GSH is present in the ER lumen, this compartment is characterised by a significantly less negative (i.e. more oxidised) GSH redox potential compared to other compartments due to the lack of GR. This oxidising environment is required for the stabilisation of intramolecular disulfide bonds formed within substrate proteins during oxidative protein folding [165]. The fate of the H_2O_2 produced during this process is currently unclear. If it remains in the ER, it could further contribute to the establishment and maintenance of an oxidising ER lumen. Alternatively, H_2O_2 might diffuse into the cytosol, where many antioxidant systems are available for its detoxification. Furthermore, H_2O_2 neutralisation within the ER cannot be excluded, as this compartment was shown to contain a glutathione peroxidase-like (GPXL)

protein [199,200]. In addition, GPXLs have also been found in the cytosol, mitochondria, chloroplasts and at the plasma membrane [199]. Although their precise functions in plants have not been fully elucidated, the ability of GPXLs to detoxify H_2O_2 and lipid peroxides is well known [201].

Because of its central location and overall interaction with all other subcellular compartments, the cytosol acts as a buffering zone for H_2O_2 and hence a multitude of antioxidant systems is present in this compartment. These include superoxide dismutase (SOD), the different components of the AsA – GSH cycle, catalase (CAT), peroxiredoxin (PRX) and GPXL proteins [86,202,203]. Since rapid changes in H_2O_2 levels can occur during physiological conditions (e.g. diurnal rhythm, temperature...) or as a result of environmental stresses (e.g. Cd pollution; Table 1), an overload of excess H_2O_2 can be translocated into and detoxified in the vacuole [88,117]. Class III peroxidases are postulated to be targeted to the vacuole [110] where they can operate in the peroxidative cycle, oxidising phenolic substrates while reducing H_2O_2. The resulting phenoxyradicals can be reduced by AsA and subsequent regeneration of DHA occurs in the cytosol [88].

Diffusion of cytosolic H_2O_2 is a major source of elevated ROS levels in the nuclear compartment, which is very sensitive to the deleterious effects of oxidation [196]. Increasing evidence suggests that oxidative signalling occurs through redox regulation of nuclear proteins, such as transcription factors, by modification of their thiol residues. The tripeptide GSH is a major soluble antioxidant that can act as a redox buffer, is important as an electron donor for multiple enzymes and has been shown to be present in the nucleus [187]. Therefore, GSH is important to detoxify an excess of nuclear H_2O_2. Its oxidised counterpart GSSG can be regenerated via GR1, which was shown to be dually targeted to the cytosol and the nucleus [196]. Since large amounts of Fe are present in the nucleus that could potentially lead to the production of harmful $^{\bullet}OH$, efficient nuclear ROS scavenging systems are crucial. Apart from GSH redox cycling, no general antioxidant enzymes, such as SOD, CAT or enzymes of the AsA-GSH cycle are present in the nucleus, but an atypical NADPH-dependent thioredoxin reductase (NTR)/TRX/PRX system has been suggested [194,196]. The nuclear 1-Cys PRX can detoxify H_2O_2 in a peroxidative way and together with the presence of NTR, this provides evidence for an antioxidant mechanism controlling oxidative events in the nucleus and contributing to the maintenance of a highly reducing environment in this compartment [196].

Because of their highly metabolic nature, oxidation events in chloroplasts, mitochondria and peroxisomes need to be tightly controlled by a diverse array of antioxidant enzymes. On one hand, H_2O_2 constitutes the majority of ROS produced in the peroxisomes as a result of GOX activity during photorespiration (Figure 1, Figure 2). Catalases are known to be important for the bulk removal of excess H_2O_2 produced in Cd-stressed plants (Table 1; [86]). Although these enzymes constitute the main

peroxisomal antioxidant mechanism [153,155], SODs and the AsA-GSH cycle are also active in this organelle [118,155,204]. On the other hand, $^1O_2$ is specifically produced in chloroplasts due to energy transfer from excited Chl to $^3O_2$ ([205]; Figure 2) and electron leakage from ETCs in both mitochondria and chloroplasts to $^3O_2$ leads to the production of $O_2^{\bullet-}$, which are the main ROS produced in these organelles (Figure 2). As both ROS species have a short half-life, they need to be rapidly detoxified by specific antioxidants, i.e. carotenoids for $^1O_2$ and specific SOD isoforms for $O_2^{\bullet-}$. As a result of SOD activities in chloroplasts and mitochondria, H_2O_2 is produced and detoxified by the AsA-GSH cycle, PRX and GPXL systems ([201,204]; Figure 2), which are known to be affected upon Cd exposure (Table 1). Furthermore, these highly reactive ROS can generate subcellular oxidation products that constitute a specific redox signature, which can be transferred to the cytosol and/or nucleus to initiate specific signal transduction pathways [90].

3.2. Metabolic organelles communicate with each other and retrograde signalling is stimulated upon Cd exposure

Under normal physiological conditions, mitochondria and chloroplasts function in energy conversion to support optimal plant growth and development. Nevertheless, upon Cd exposure, cellular resources need to be properly used for these organelles to maintain their primary function on one hand and to invest in acclimation responses on the other hand. To ensure plant acclimation to Cd stress, communication between different organelles is crucial and ROS play a significant role in this process. On one hand, direct exchange of substrates or ROS through membrane contact sites (MCS) has been observed and might be important to optimally regulate cell metabolism under adverse conditions (for a review, see [206]). However, this research field is still in its infancy. On the other hand, inter-organellar cross-talk via retrograde (organelle-to-nucleus) and anterograde (nucleus-to-organelle) signalling has been subject of multiple studies and is described in more detail in many reviews [126,207–213]. Retrograde signalling functions in developmental processes such as organelle biogenesis but is also activated in response to stress conditions to achieve stress acclimation [117,211]. Retrograde signals can be diverse in origin, consisting of different biomolecules, such as RNA, ROS, proteins and metabolites [211], as well as oxidation and/or degradation products derived from macromolecules, such as pigments and lipids [125]. Whereas multiple pathways have been described for chloroplast retrograde signalling [125,213,214], it is currently unclear whether they are involved in responses to Cd exposure. Although research into peroxisomal retrograde signalling is still in its infancy, a recent meta-analysis revealed a set of genes that are co-expressed in response to various conditions altering peroxisomal ROS metabolism, suggesting a key role for peroxisome-derived ROS in

transcriptional regulation [215]. Hence, it will be interesting to unravel the involvement of chloroplast and peroxisomal retrograde signalling in plant responses to Cd stress in future studies. In mitochondrial retrograde signalling, a key role has been described for the ER-residing NAC family transcription factors ANAC013 and ANAC017 [216,217]. It has been shown that Cd exposure disturbs mitochondrial function and causes a switch from electron transfer through the ETC to alternative respiration via AOX [147]. *AtAOX1a* is a mitochondrial dysfunction stimulon gene, which contains a mitochondrial dysfunction motif in its promoter and is upregulated in response to mitochondrial stress (potentially caused by mitochondrial ROS) [217]. Hence, the induction of *AOX1a* during Cd stress in *A. thaliana* [147] suggests that such retrograde signalling also occurs in response to this metal. A clear interplay has been observed between ROS, nitric oxide (NO) and ethylene in *AOX1a* induction in Cd-stressed plants. Indeed, increases in *AOX* gene expression, AOX protein levels and alternative respiration rate in *H. vulgare* roots exposed to 150 µM Cd were further elevated by addition of the NO donor sodium nitroprussid (SNP) or H_2O_2 [36,151]. In contrast, *AOX1a* upregulation in WT Arabidopsis seedlings exposed to 5 µM Cd, was reduced in the *acs2-1acs6-1* ethylene biosynthesis mutant and the *ein2-1* ethylene-insensitive mutant [218].

3.3. OXI1 functions as an integrator of the cellular redox state with ethylene signalling under Cd stress (Figure 3)

The typical stress response curve consists of an initial response phase followed by an acclimation phase during which a new steady state is reached [219]. The first phase is defined by a decline in one or multiple physiological functions, followed by early signalling events to activate coping mechanisms to avoid acute damage. These counter-reactions define the restitution phase and may initiate long-term acclimation. However, if the plant is overloaded and acclimation does not occur, the system becomes exhausted and irreversible damage is inevitable [8]. In this regard, GSH levels can be considered an intrinsic feature of the early Cd-induced stress response, as an immediate GSH depletion in roots of *A. thaliana* seedlings occurs [8] due to allocation to PC synthesis [7]. Nevertheless, no GSH oxidation is observed in response to acute Cd stress and it was suggested that GSH depletion prevents its oxidation and is sufficient to alter the cellular redox potential and drive GSH accumulation [8]. Even under GSH-limiting conditions in the *cad2* mutant, Cd-induced GSH depletion due to PC production does not alter the oxidation state of the GSH pool [7,82,183]. Together with an immediate decrease in GSH levels, Cd exposure causes a rapid increase in *RBOH* gene expression [8], possibly initiating a ROS signalling cascade. In this regard, the presence of extracellular GSH is important as it might be oxidised by the NADPH oxidase-generated ROS and support the maintenance of the apoplastic redox balance. Here, GSSG cannot be regenerated as no GR is localised in the apoplast. However, the activation of the γ-

glutamyl cycle, mainly driven by the apoplastic γ-glutamyl transferase (GGT) enzyme, which gene expression is rapidly induced upon Cd exposure and which catalyses the transfer of the γ-glutamyl group of GSH to a range of acceptors, prevents the accumulation of GSSG in the apoplastic space, mitigating oxidative stress [8]. The alterations in apoplastic GSH redox state and ROS production might serve as an initial ROS signal to ensure plant acclimation to Cd stress.

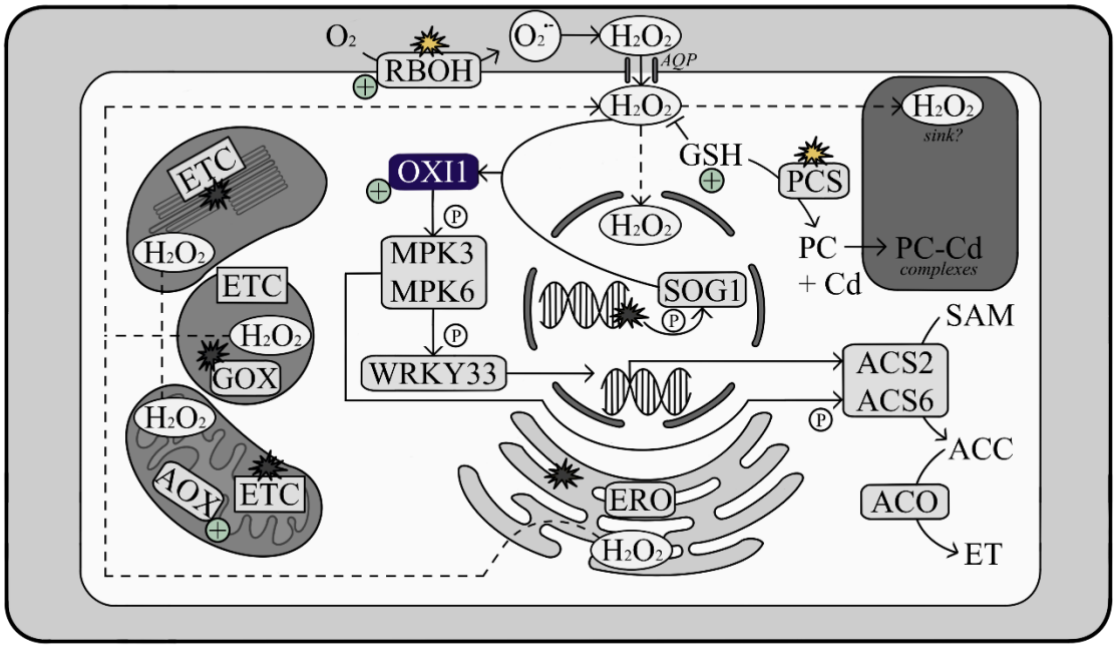

Figure 3: A model depicting Cd-induced H_2O_2 signalling and its proposed interaction with ethylene signalling. Oxidative signal-inducible kinase 1 (OXI1) is suggested as a central integrator of cellular H_2O_2 signals with downstream responses. Cadmium-induced increases in cellular reactive oxygen species (ROS) levels, originating from different subcellular compartments (see Figure 1 and section 3.3), are perceived by OXI1, which in its turn activates mitogen-activated protein kinase 3 (MPK3) and MPK6. These kinases stabilise 1-aminocyclopropane 1-carboxylic acid (ACC) synthase 2 (ACS2) and ACS6 via phosphorylation at the protein level or indirectly increase their transcription via the transcription factor WKRY33. Next, ACC oxidase converts ACC into ethylene, inducing ethylene signalling. Solid arrows indicate confirmed reactions; dashed arrows are postulated. Abbreviations: alternative oxidase (AOX), 1-aminocyclopropane-1-carboxylic acid (ACC), ACC oxidase (ACO), ACC synthase (ACS), aquaporins (AQP), endoplasmic reticulum oxidoreductin (ERO), electron transport chain (ETC), ethylene (ET), glutathione (GSH), glycolate oxidase (GOX), hydrogen peroxide (H_2O_2), mitogen-activated protein kinase (MPK), oxidative signal-inducible kinase1 (OXI1), phytochelatin (PC), phytochelatin synthase (PCS), respiratory burst oxidase homolog (RBHOH), suppressor of gamma response 1 (SOG1; transcription factor), WRKY DNA-binding protein 33 (WRKY33; transcription factor).

Upon its translocation to the cytosol via aquaporins, H_2O_2 triggers an intracellular signalling cascade. Within the cytosol, H_2O_2 is perceived by OXI1, which in turn activates mitogen-activated protein kinase 3 (MPK3) and MPK6 [220]. Genes encoding OXI1 and these MPKs have been shown to be induced upon Cd exposure in *A. thaliana* [191,193,221]. Both MPK3 and MPK6 phosphorylate the transcription factor WRKY33 and the 1-aminocyclopropane-1-carboxylic acid (ACC) biosynthesis enzymes, ACS2 and ACS6, that become more stable upon phosphorylation [222]. In addition, WRKY33 induces the expression of genes encoding both enzymes, which indicates that ACC biosynthesis is regulated at the transcriptional as well as posttranslational level. Indeed, also ACC production is immediately stimulated upon Cd exposure [8]. Since ACC is a direct precursor of the stress hormone ethylene, also a Cd-induced ethylene production is observed that strongly depends on ACS2 and ACS6 activity in *A. thaliana* [223]. Similarly, Cd-induced ethylene production was also reported in other plant species including *P. sativum* [224,225], *T. aestivum* [226], *L. chinensis* [227] and *B. juncea* [228]. A reciprocal interaction between GSH and ethylene signalling in Cd-induced responses was demonstrated, since restoration of GSH levels after their initial depletion was delayed in the Arabidopsis ethylene-insensitive *ein2-1* mutant [221]. On the other hand, Cd-induced ACC production was even further stimulated in the GSH-deficient *cad2* mutant [183].

Together, these data clearly demonstrate that OXI is a key player in ROS signalling and lies at the heart of ethylene production and signalling under Cd stress. Moreover, its expression is induced by SOG1 [189], which plays an important role in Cd-induced oxidative stress responses and the DDR [18]. Furthermore, OXI1 plays a potential role in retrograde signalling from the chloroplast [125] and, together with AOX1a, belongs to the set of mitochondrial dysfunction stimulus genes that are important in mitochondrial retrograde signalling [217]. Interestingly, *AOX1a* and *OXI1* gene expression are also stimulated in Cd-exposed *A. thaliana* seedlings. Taken together, we postulate that OXI1 acts as a central integrator of ROS signals derived from multiple subcellular compartments with other important signals (e.g. GSH, ethylene, DDR) required for plant acclimation to Cd stress.

Conclusion

Upon exposure to Cd stress, GSH depletion as a consequence of PC biosynthesis and activation of NADPH oxidases rapidly induce alterations in cellular redox homeostasis. Furthermore, Cd enhances ROS production in several subcellular compartments. Sensing of H_2O_2 by OXI1 triggers a signalling cascade that connects ROS perception to other intracellular signals such as ethylene. Furthermore, ROS can serve a role in communication between different organelles, allowing for the coordination of cellular stress responses, ultimately determining cell fate. Whether cells are able to survive Cd stress depends on many factors, including the type of ROS produced, the timing of ROS production, their concentration and their subcellular localisation. This in turn strongly depends on Cd speciation, bioavailability, concentration and exposure duration. Furthermore, subcellular localisation of Cd after its uptake in plants is also a key determinant of Cd-induced stress responses. Therefore, it is essential to focus on the temporal and spatial resolution of Cd-induced ROS production, oxidised targets and antioxidants in combination with Cd localisation in future studies to better understand Cd-specific ROS signatures.

Figure and table legends

Figure 1: Overview of subcellular ROS production in plant cells under physiological and Cd-exposed conditions. When taken up in plant cells, Cd is immediately complexed with phytochelatins (PC) at the expense of cellular glutathione (GSH). The resulting Cd-PC complexes are sequestered in the vacuole. As Cd is not redox-active, it cannot directly induce ROS production. However, it indirectly enhances ROS levels through displacement of essential redox-active metals, such as Fe and Cu. These in turn catalyse Fenton and Haber-Weiss reactions (top figure) leading to the production of highly reactive hydroxyl radicals ($^{\bullet}OH$) in multiple subcellular compartments. The subcellular redox state is highlighted with a colour gradient ranging from red (more oxidising) to blue (more reducing). Whereas the metabolic organelles (chloroplasts, mitochondria, peroxisomes) are the most reducing, the endoplasmic reticulum and apoplast are most oxidising and the cytosol acts as an intermediate buffering zone. Arrows indicate transfer of ROS (mainly hydrogen peroxide; H_2O_2) between subcellular compartments (solid arrows: confirmed; dashed arrows: postulated). Abbreviations: aquaporins (AQP), endoplasmic reticulum oxidoreductin (ERO), electron transport chain (ETC), glutathione (GSH), glycolate oxidase (GOX), hydrogen peroxide (H_2O_2), hydroxide ion (OH^-), hydroxyl radical ($^{\bullet}OH$), superoxide ($O_2^{\bullet-}$), phytochelatin (PC), phytochelatin synthase (PCS), respiratory burst oxidase homolog (RBHOH).

Figure 2: Major subcellular ROS production and detoxification mechanisms in plants. Compartments are categorised based on their oxidation state. Abbreviations: hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), superoxide ($O_2^{\bullet-}$), ascorbic acid (AsA), catalase (CAT), endoplasmic reticulum oxidoreductin (ERO), electron transport chain (ETC), glycolate oxidase (GOX), glutathione (GSH), glutathione peroxidase-like (GPXL), peroxiredoxin (PRX), superoxide dismutase (SOD).

Figure 3: A model depicting Cd-induced H_2O_2 signalling and its proposed interaction with ethylene signalling. Oxidative signal-inducible kinase 1 (OXI1) is suggested as a central integrator of cellular H_2O_2 signals with downstream responses. Cadmium-induced increases in cellular reactive oxygen species (ROS) levels, originating from different subcellular compartments (see Figure 1 and section 3.3), are perceived by OXI1, which in its turn activates mitogen-activated protein kinase 3 (MPK3) and MPK6. These kinases stabilise 1-aminocyclopropane 1-carboxylic acid (ACC) synthase 2 (ACS2) and ACS6 via phosphorylation at the protein level or indirectly increase their transcription via the transcription factor WKRY33. Next, ACC oxidase converts ACC into ethylene, inducing ethylene signalling. Solid arrows indicate confirmed reactions; dashed arrows are postulated. Abbreviations: alternative oxidase

(AOX), 1-aminocyclopropane-1-carboxylic acid (ACC), ACC oxidase (ACO), ACC synthase (ACS), aquaporins (AQP), endoplasmic reticulum oxidoreductin (ERO), electron transport chain (ETC), ethylene (ET), glutathione (GSH), glycolate oxidase (GOX), hydrogen peroxide (H_2O_2), mitogen-activated protein kinase (MPK), oxidative signal-inducible kinase1 (OXI1), phytochelatin (PC), phytochelatin synthase (PCS), respiratory burst oxidase homolog (RBHOH), suppressor of gamma response 1 (SOG1; transcription factor), WRKY DNA-binding protein 33 (WRKY33; transcription factor).

Table 1: Cadmium-induced ROS production and scavenging in plants. The table catalogues research articles published over the last 5 years (since 2018) that report Cd-induced ROS production and effects on both antioxidant enzymes (transcript levels and/or activity) and metabolites (transcript levels/activity of biosynthetic enzymes and/or concentrations).

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