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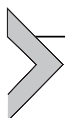

CHAPTER SIX

Molecular and Cellular Aspects of Contaminant Toxicity in Plants: The Importance of Sulphur and Associated Signalling Pathways

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Abstract

Environmental contamination with metals and organic compounds poses a serious threat to human health. Investigating plant responses to these contaminants at the molecular and cellular level is crucial to optimize phytoremediation strategies to clean up contaminated soils. Two key players in plant stress responses are the

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sulphur-containing amino acids cysteine and methionine. Cysteine is an important constituent of the metal-chelating metallothioneins and is also the precursor for glutathione and subsequent phytochelatin synthesis. During stress conditions, glutathione is involved in (1) metal chelation, (2) xenobiotic detoxification and (3) antioxidative defence. The activated form of methionine, S-adenosylmethionine, is involved in the synthesis of ethylene and polyamines, both playing important roles in signal transduction. This review provides an overview of sulphur uptake and assimilation and its conversion into basic metabolites essential for detoxification and signal transduction during metal and organic contaminant exposure in plants. Furthermore, the cross talk between these pathways and their relation to the contaminant-induced oxidative challenge are discussed.

1. GENERAL INTRODUCTION

Since the industrial revolution, but especially over the past few decades, abiotic contaminants – mainly from agricultural and industrial origin – have emerged into the environment and are recognized to pose a serious threat to the environment and human health (Ibrahim, Hayyan, AlSaadi, Hayyan, & Ibrahim, 2016; Noguera-Oviedo & Aga, 2016). These contaminants can be categorized into different groups based on their physicochemical properties, but their persistence (e.g., metals) and degradability (e.g., organics) are determining factors for their presence in the environment. As a result, different strategies are needed to remediate the environment. Microbe-assisted phytoremediation is a promising technology to be implemented in the cleanup of metal-polluted soils and organics in soil and air (chapter: Phytoremediation and Phytomining: Status and Promise by Chaney & Baklanov, 2017; Chirakkara, Cameselle, & Reddy, 2016; chapters: Mycorrhiza-Assisted Phytoremediation by Coninx, Martinova, & Rineau, 2017; Bio- and Phytoremediation of Pesticide Contaminated Environments: A Review by Eevers, White, Vangronsveld, & Weyens, 2017; Plants in Air Phytoremediation by Gawronski, Gawronska, Lomnicki, Sæbo, & Vangronsveld, 2017; Gerhardt, Huang, Glick, & Greenberg, 2009; chapter: Potential Role of Plant-Associated Bacteria in Plant Metal Uptake and Implications in Phytotechnologies by Kidd, Alvarez-Lopez, Becerra-Castro, Cabello-Conejo, & Prieto-Fernández, 2017; Ma, Oliveira, Freitas, & Zhang, 2016; Vangronsveld et al., 2009). However, to exploit this technology, it is important to unravel the underlying molecular and cellular responses of plants to minimize phytotoxic responses.

Besides growth reduction and retardation, morphological and physiological responses as a result of organics or metal stress have been extensively described in plants (Verkleij, Golan-Goldhirsh, Antosiewisz,

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Schwitzguébel, & Schröder, 2009). Multiple studies have focused on the underlying molecular mechanisms of these stress responses. To cope with toxic compounds, plants rely on different detoxification mechanisms such as (1) biotransformation, (2) conjugation and (3) sequestration, all of which should be highly coordinated to prevent cellular damage. In addition to the detoxification of excess contaminants, other factors such as environmental conditions (e.g., temperature, humidity) determine the stress intensity perceived by the plant (Reichenauer & Germida, 2008). Pivotal modulators of plant metabolism upon multiple environmental challenges are reactive oxygen species (ROS), which are involved in oxidative damage as well as signalling. In response to the alteration of ROS levels, metabolic adjustments lead to a newly established cellular homeostasis essential for plant performance (Cuypers et al., 2016; Foyer & Noctor, 2005).

When considering cellular detoxification and regulation mechanisms, the sulphur-containing amino acids, cysteine and methionine, play an important role. Cysteine is a major constituent of the metal chelators metallothioneins (MTs), glutathione (GSH) and phytochelatins (PCs) (Anjum, Gill, & Gill, 2014). MTs are gene-encoded peptides synthesized by ribosomes, whereas GSH and PCs are metabolically synthesized (Anjum et al., 2015). In addition to its function in metal chelation, GSH is often consumed in conjugation reactions of organic compounds before their sequestration into the vacuole (Dixon, Skipsey, & Edwards, 2010). Besides its role in detoxification, GSH is a major cellular antioxidant in plants (Noctor et al., 2012). As such, it is clear that detoxification and regulation are closely interconnected in plant stress acclimation. Methionine, the other sulphur-containing amino acid, forms the basis of S-adenosylmethionine (SAM), a central component in plant metabolism. It is also the initial biosynthetic compound in the production of the stress hormone ethylene (Sauter, Moffatt, Saechao, Hell, & Wirtz, 2013). Besides ROS, ethylene is an important regulator of stress perception and is known to be involved in multiple responses induced by abiotic stress (Keunen, Schellingen, Vangronsveld, & Cuypers, 2016). In consequence, when plants are exposed to organic contaminants or metals, sulphur is an essential element in (1) detoxification pathways and (2) the regulation of stress responses. Therefore, this review focusses on sulphur uptake and assimilation and its conversion into basic metabolites essential for detoxification and regulation mechanisms during organic contaminant and metal exposure in plants. These processes are strongly interconnected and are discussed in view of the oxidative challenge and signal transduction during stress conditions.

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2. SULPHUR UPTAKE AND ASSIMILATION

Sulphur is an essential macronutrient for all organisms, serving different metabolic functions. It is incorporated into the amino acids cysteine and methionine, controlling the structure and biological activity of many proteins (Davidian & Kopriva, 2010; Gotor et al., 2015). Furthermore, it is an important component of several coenzymes and prosthetic groups and is involved in plant responses to both biotic and abiotic stress factors (Davidian & Kopriva, 2010; Gigolashvili & Kopriva, 2014; Romero et al., 2014). Sulphur present as inorganic sulphate ($SO_4{}^{2-}$) in soils is uptaken by plants. To enter metabolic pathways, sulphate is assimilated in plants through a pathway consisting of (1) sulphate uptake, (2) sulphate activation, (3) sulphate reduction and (4) synthesis of cysteine (Fig. 1) (Droux, 2004; Ravilious & Jez, 2012).

Sulphate is uptaken by plants via the action of proton/sulphate cotransport systems (Gigolashvili & Kopriva, 2014; Takahashi, Kopriva, Giordano, Saito, & Hell, 2011). The plant family of sulphate transporters (SULTRs), composed of 12–16 genes depending on the plant species, is subdivided into four functional groups based on their sequence, biochemical characteristics and physiological function (Takahashi et al., 2011). The SULTR family is best characterized in the model organism *Arabidopsis thaliana* (Gigolashvili & Kopriva, 2014). The most important transporters involved in sulphate uptake in roots are the high-affinity SULTR1;1 and SULTR1;2 proteins (group 1). However, as the expression of the *SULTR1;1* gene in roots is much lower than that of *SULTR1;2*, the latter is considered the most important transporter involved in plant sulphate uptake during normal sulphate supply (Gigolashvili & Kopriva, 2014; Rouached et al., 2008). During sulphate starvation, however, *SULTR1;1* expression is strongly upregulated, a response that is likely due to the presence of a sulphur-responsive (SURE) *cis* element in its promoter (Maruyama-Nakashita et al., 2005). Another known SULTR is SULTR1;3, which is localized in phloem companion cells and involved in sulphate transport from source to sink (Yoshimoto, Inoue, Saito, Yamaya, & Takahashi, 2003). Furthermore, the low-affinity group 2 SULTRs facilitate long-distance sulphate transport (Gigolashvili & Kopriva, 2014; Takahashi et al., 2000). The activity of SULTR2;1 is modulated by SULTR3;5, which does not transport sulphate itself (Kataoka, Hayashi, Yamaya, & Takahashi, 2004). In contrast to SULTR3;5, other group 3 SULTRs function in sulphate import into plastids (Cao et al., 2013; Gigolashvili & Kopriva,

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2014), as the enzymes necessary for sulphate reduction are exclusively present in these organelles (Davidian & Kopriva, 2010). Transporters belonging to group 4 are localized in the tonoplast, releasing sulphate from vacuoles (Kataoka, Watanabe-Takahashi, et al., 2004; Takahashi et al., 2011).

After its uptake in plant cells, sulphate is activated in an adenylation reaction catalyzed by ATP sulphurylase (ATPS). The resulting adenosine 5′-phosphosulphate (APS) is an important branching point in the sulphate assimilation pathway (Fig. 1). It can be phosphorylated by the action of APS kinase (APK), leading to the formation of 3′-phosphoadenosine 5′-phosphosulphate (PAPS), an important activated sulphate donor for many sulphation reactions in plant secondary metabolism. In the primary sulphate assimilation pathway, however, APS reductase (APR) reduces APS to sulphite ($SO_3{}^{2-}$), which is further reduced to sulphide (S^{2-}) by the ferredoxin-dependent sulphite reductase (SiR). Sulphide is subsequently used for the synthesis of cysteine (Droux, 2004; Takahashi et al., 2011). Whereas ATPS is also present in the cytosol, the reductive steps of the sulphate assimilation pathway only take place in plastids (Davidian & Kopriva, 2010).

The final step in the assimilation pathway of reduced sulphate is the synthesis of cysteine (Fig. 1). This sulphur-containing amino acid is subsequently incorporated into different compounds including MTs, GSH and PCs, important in metal chelation, detoxification of xenobiotics and antioxidative defence (Anjum et al., 2014). Furthermore, cysteine is an important building block for the synthesis of methionine, the second sulphur-containing amino acid (Wirtz & Droux, 2005). In the first and rate-limiting step of cysteine biosynthesis, O-acetylserine (OAS) is synthesized from L-serine and acetyl coenzyme A by serine acetyl transferase (SAT) (Wirtz & Droux, 2005; Wirtz & Hell, 2006). Subsequently, cysteine is formed by substitution of the acetate of OAS with sulphide in a β-replacement reaction catalyzed by OAS (thiol) lyase (OASTL). Both SAT and OASTL are present in the cytosol, plastids and mitochondria (Anjum et al., 2014). These enzymes were demonstrated to associate, forming the hetero-oligomeric cysteine synthase complex. This association with OASTL is necessary for the stability and activity of SAT, which is otherwise subjected to feedback inhibition by cysteine. In contrast, OASTL activity is silenced when bound to SAT, suggesting that this enzyme only functions as a chaperone-like subunit in the cysteine synthase complex (Wirtz et al., 2010; Wirtz & Hell, 2006). However, as cellular OASTL activity is 100−300 times higher than SAT activity, free OASTL enzymes

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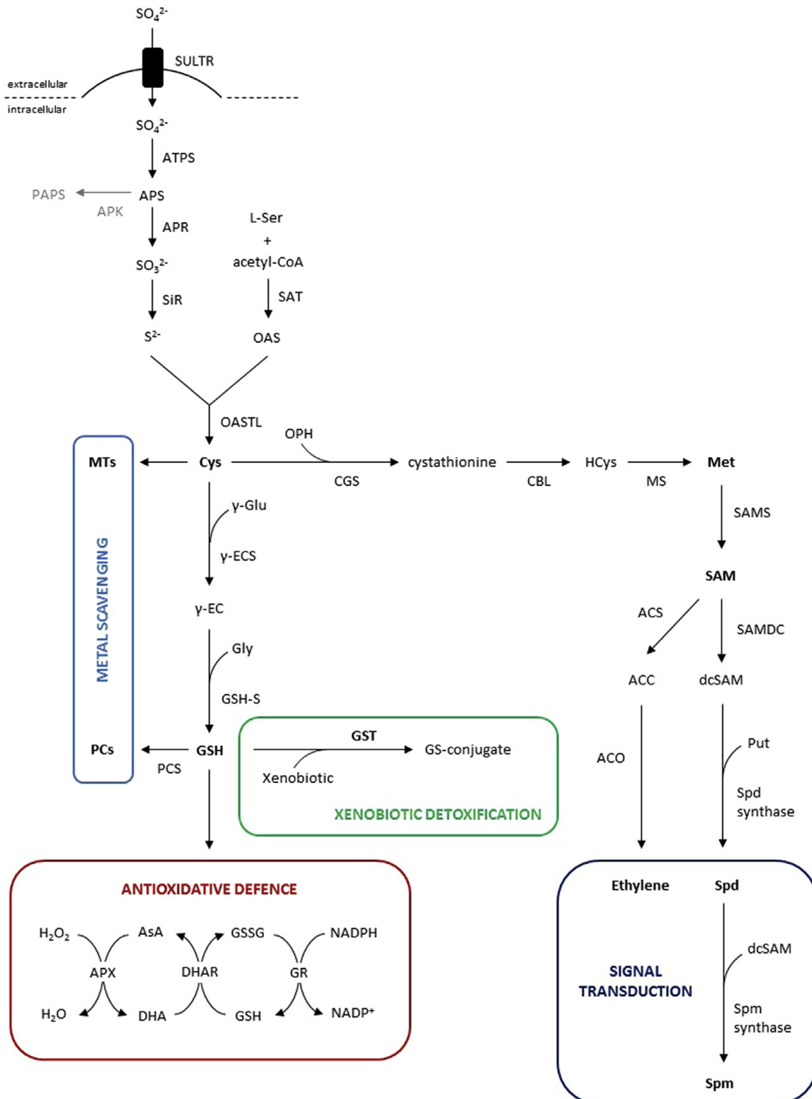

Figure 1 ***Biosynthesis pathway of sulphur-derived cellular compounds and their involvement in metal scavenging, xenobiotic detoxification, antioxidative defence and signal transduction.*** *γ-EC*, γ-glutamylcysteine; *γ-ECS*, γ-EC synthetase; *γ-Glu*, γ-glutamate; *ACC*, 1-aminocyclopropane-1-carboxylic acid; *acetyl-CoA*, acetyl coenzyme A; *ACO*, ACC oxidase; *ACS*, ACC synthase; *APK*, APS kinase; *APR*, APS reductase; *APS*, adenosine 5′-phosphosulphate; *APX*, ascorbate peroxidase; *AsA*, antioxidative ascorbate; *ATPS*, ATP sulphurylase; *CBL*, cystathionine β-lyase; *CGS*, cystathionine γ-synthase; *Cys*, cysteine; *dcSAM*, decarboxylated SAM; *DHA*, dehydroascorbate; *DHAR*, DHA reductase; *Gly*, glycine; *GR*, glutathione reductase; *GS-conjugate*, glutathione S-conjugate; *GSH*,

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are present in cells and catalyze the formation of cysteine from sulphide and OAS released by the complex (Droux, 2004; Saito, 2000). It must be noted that several metabolites regulate the stability of the cysteine synthase complex. During sulphur-deprived conditions, for example, increased OAS levels stimulate dissociation of the complex, thereby inhibiting SAT activity and further OAS accumulation. In contrast, the presence of sulphide counteracts the dissociation of SAT and OASTL, resulting in the maintenance of SAT activity as long as sulphide availability is not a limiting factor (Droux, Ruffet, Douce, & Job, 1998; Takahashi et al., 2011; Wirtz & Hell, 2006).

2.1 Responses to Metal Stress

In addition to its regulation by internal stimuli, the sulphate assimilation pathway is affected by environmental factors including metal exposure (Ernst, Krauss, Verkleij, & Wesenberg, 2008; Na & Salt, 2011). Indeed, several metals were shown to influence the expression of genes encoding both high- and low-affinity SULTRs, thereby affecting sulphate uptake and assimilation. Nocito et al. (2006), for example, demonstrated that exposure to different concentrations of cadmium (Cd), copper (Cu) and zinc (Zn) increased the expression of *ST1;1*, a high-affinity SULTR in *Zea mays*. As a result, sulphate uptake capacity in roots was significantly enhanced during metal exposure (Nocito et al., 2006). Furthermore, Zn was shown to enhance root *SULTR1;1*, *SULTR1;2*, *SULTR4;1* and *SULTR4;2* expression and sulphate uptake in *Brassica pekinensis* (Stuiver et al., 2014). Similarly, Dixit et al. (2016) demonstrated an upregulation of *SULTR1;1*, *SULTR1;2 SULTR2;2* and *SULTR4;1* expression in roots of *Oryza sativa* exposed to the metalloid arsenic (As), resulting in increased sulphur accumulation and translocation. As a consequence, cysteine, GSH and PC levels were higher in both roots and shoots (Dixit et al., 2016). However, exposure to chromium (Cr) clearly decreased the expression

glutathione; *GSH-S*, glutathione synthetase; *GSSG*, glutathione disulphide; *GST*, glutathione transferase; H_2O, water; H_2O_2, hydrogen peroxide; *HCys*, homocysteine; *L-Ser*, L-serine; *Met*, methionine; *MS*, methionine synthase; *MT*, metallothionein; *OAS*, O-acetylserine; *OASTL*, OAS (thiol) lyase; *OPH*, O-homophosphoserine; *PAPS*, 3′-phosphoadenosine 5′-phosphosulphate; *PC*, phytochelatin; *PCS*, phytochelatin synthase; *Put*, putrescine; S^{2-}, sulphide; *SAM*, S-adenosylmethionine; *SAMDC*, SAM decarboxylase; *SAMS*, SAM synthetase; *SAT*, serine acetyl transferase; *SiR*, sulphite reductase; SO_3^{2-}, sulphite; SO_4^{2-}, sulphate; *Spd*, spermidine; *Spm*, spermine; *SULTR*, sulphate transporter.

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levels of *ST1;1* and significantly suppressed sulphate uptake in *Z. mays* (Schiavon et al., 2007). Cr was also shown to reduce sulphate uptake and negatively affect the transcription of several SULTRs, including *SULTR1;3*, *SULTR2;1*, *SULTR3;2*, *SULTR3;5* and *SULTR4;1*, in roots of *Brassica juncea* after 24 h of exposure, whereas expression levels of *SULTR4;2* were significantly increased (Schiavon et al., 2012).

In addition to their effect on sulphate uptake, metals also influence transcript levels, protein abundance and activity of enzymes catalyzing different reactions in the sulphate assimilation pathway. For example, an increased activity of the sulphate-activating enzyme ATPS was reported in leaves of *A. thaliana* exposed to 50 μM Cd for 3 or 5 days (Bashir, Ahmad, Bagheri, Nauman, & Qureshi, 2013). Similar effects were demonstrated in *Sedum alfredii*, with Cd increasing root and shoot *ATPS* transcript levels in a concentration-dependent manner. Moreover, the expression of the *SAT* gene, encoding the first enzyme in cysteine biosynthesis, was also significantly upregulated by Cd exposure in the roots (Liang et al., 2014). In addition to Cd, Cu was shown to affect sulphate assimilation, as demonstrated by a strongly increased *ATPS* expression in roots of both *A. thaliana* and *Arabidopsis halleri* (Weber, Trampczynska, & Clemens, 2006). Furthermore, Cu-induced increases in the protein abundance of APR, SAT and OASTL in *O. sativa* roots were reported by Song et al. (2013). By affecting enzymes involved in sulphate assimilation, several metal(loid)s including As (Dixit et al., 2016; Talukdar & Talukdar, 2014), Cd (Bashir et al., 2013; Liang et al., 2014; Nocito et al., 2006), Cr (Schiavon et al., 2012; Schiavon, Pilon-Smits, Wirtz, Hell, & Malagoli, 2008), Cu (Gajewska, Glowacki, Mazur, & Sklodowska, 2013), nickel (Ni) (Gajewska et al., 2013), lead (Pb) (Mandavian, Ghaderian, & Schat, 2016) and Zn (Stuiver et al., 2014) were shown to alter cysteine levels in a wide range of plant species.

However, it is important to note that plant responses to metal exposure depend on sulphate supply, as metal-induced effects observed during sulphate-deprived conditions often differ from those observed during normal sulphate availability. Results of a study by Bashir et al. (2015) indicate that under conditions of sulphate starvation, *B. juncea* displayed higher Cd-induced oxidative stress levels and an increased Cd sensitivity. Furthermore, sulphur addition was reported to increase Cd uptake in roots of *Brassica chinensis*, while inhibiting root-to-shoot translocation of the metal. Although the addition of sulphate to the growth medium increased plant Cd uptake, it also relieved the Cd-induced inhibition of root and shoot growth and significantly reduced malondialdehyde (MDA) and superoxide ($O_2^{\bullet-}$) levels in both organs (Liang

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et al., 2016). Decreases in *Corchorus olitorius* dry weight induced by aluminium (Al), Cd, Cu and Pb were alleviated by the addition of either K_2SO_4 or cysteine to the growth medium (Mazen, 2004). These results are of particular interest in the context of phytoremediation, as this soil remediation strategy strongly benefits from plants exhibiting a high metal uptake capacity and increased metal tolerance.

The involvement of sulphate uptake and assimilation in plant responses to metal stress is further supported by the observation that modification of genes involved in sulphate assimilation alters plant tolerance to a broad array of metals. Indeed, overexpression of *SAT* was shown to increase OAS, cysteine and GSH levels in *A. thaliana*, thereby increasing its resistance to Ni-induced growth inhibition (Freeman et al., 2004). Furthermore, overexpression of different *OASTL* isoforms increased Cd tolerance of both *A. thaliana* and *Nicotiana tabacum* (Dominguez-Solis, Gutierrez-Alcala, Romero, & Gotor, 2001; Dominguez-Solis et al., 2004; Harada, Choi, Tsuchisaka, Obata, & Sano, 2001; Kawashima et al., 2004; Ning, Zhang, Yao, & Yu, 2010). Interestingly, transgenic *Brassica napus* plants overexpressing *miR395* also showed diminished Cd-induced oxidative stress levels and were more tolerant to Cd. This miRNA is induced by sulphate starvation and controls the expression levels of *SULTR2;1* and three *ATPS* isoforms. Expression of *miRNA395* is also increased during Cd exposure, highlighting the similarity between plant responses to both stress factors (Gielen, Remans, Vangronsveld, & Cuypers, 2016; Zhang, Song, Shu, Zhang, & Yang, 2013).

The importance of sulphate assimilation in plant responses to metal stress is supported by the observation that transcript levels and activity of enzymes involved in sulphate uptake often differ between metal hyperaccumulators and their nonaccumulator counterparts. For example, Freeman et al. (2004) reported strong positive correlations between OAS, cysteine and GSH levels and shoot Ni concentrations in various *Thlaspi* hyperaccumulators and nonaccumulators. Indeed, the metal-accumulating species *Thlaspi goesingense* displayed increased SAT activity and OAS, cysteine and GSH levels in comparison to the genetically related nonaccumulator *A. thaliana* under control conditions. When exposed to Ni, this resulted in an increased resistance of the former species against Ni-induced oxidative stress, as indicated by considerably lower lipid peroxidation levels (Freeman et al., 2004). Metal-tolerant plants of the same species often exhibit increased sulphate assimilation capacities when compared to their metal-sensitive counterparts. For example, an As-tolerant *Lens culinaris* cultivar showed strongly increased *SAT* and *OASTL* transcript levels and activities and significantly enhanced cysteine and GSH

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levels when compared to an As-sensitive cultivar under As exposure conditions. Interestingly, As differently affected transcript levels of group 1 SULTRs between both cultivars, with *SULTR1;1* and *SULTR1;2* upregulation in the tolerant cultivar and downregulation in the sensitive cultivar after 24 h of exposure (Talukdar & Talukdar, 2014). Similarly, Song et al. (2013) reported more pronounced Cu-induced increases in SAT and OASTL protein abundance in a Cu-tolerant *O. sativa* variety as compared to a Cu-sensitive variety. Furthermore, the induction of ATPS and OASTL activities by Cd exposure in a tolerant *B. chinensis* cultivar was less pronounced or even absent in its sensitive counterpart (Liang et al., 2016). In conclusion, it is obvious that sulphate uptake and assimilation play a prominent role in plant responses to metal stress. This can be explained by the crucial role of cysteine-containing compounds in metal chelation and detoxification, as discussed in Section 3. Therefore, metallophytes could be useful in phytoremediation technologies (chapter: Metallophytes of Serpentine and Calamine Soils – Their Unique Ecophysiology and Potential for Phytoremediation by Wójcik et al., 2017), which might be further enhanced by plant-associated bacteria and fungi (chapter: The Bacterial and Fungal Microbiota of Hyperaccumulator Plants: Small Organisms, Large Influence by Thijs, Langill, & Vangronsveld, 2017).

2.2 Responses to Organic Contaminants

Although our knowledge on the effects of organic environmental contaminants on sulphate assimilation is scarce, the effects of herbicides and safeners – used to selectively protect crops from herbicide damage – have been frequently described (Abu-Qare & Duncan, 2002; Hirase & Molin, 2003). In 1985, the Lamoureux Laboratory described that pretreatment of *Z. mays* with low levels of the chloroacetamide herbicide 2-chloro-N,N-di-2-propenylacetamide protected them from later exposure to higher concentrations of the herbicide by increasing GSH synthesis (Ezra, Rusness, Lamoureux, & Stephenson, 1985). Similarly, Adams, Blee, and Casida (1983) showed that dichloroacetamide herbicide antidotes enhanced sulphate metabolism in *Z. mays* roots. Both dichlormid and benoxacor induced ATPS, whereas flurazole acted on OASTL (Hirase & Molin, 2003). Such an effect was also observed a few years later for *Sorghum bicolor* by Gronwald, Fuerst, Eberlein, and Egli (1987). In 1990, evidence for a direct interaction of N,N-diallyl-2,2-dichloroacetamide and 4-dichloroacetyl-3,4-dihydro-3-methyl-2H-1,4-benzo-oxazine (CGA 154 281) with sulphate assimilation and GSH concentrations was presented in *Z. mays* (Farago & Brunold,

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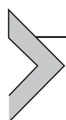
1990). Similar to the safener CGA 154 281, cysteine formation and GSH synthesis were also induced by the safener 1-dichloroacetyl-hexahydro-3,3,8-δα-trimethyl-pyrrolo-[1,2-α]-pyrimidine-6-(2H)-one-(dicyclonone) (BAS 145 138) in *Z. mays* (Kocsy et al., 2001). These specific effects of safeners on sulphate assimilation have been attributed to their influence on oxidative stress levels, but are not completely unravelled yet. Recently, the effect of three herbicide safeners (mefenpyr-diethyl, fenchlorazole-ethyl and dichlormid) on the content of sulphur-containing metabolites in Fe-deficient barley was investigated (Bartucca et al., 2016). All three safeners effectively induced ATPS activity, but the effect on OASTL activity was dependent on concentration as well as exposure time. An initial reduction of OASTL activity was followed by a strong induction. The authors speculated that safeners initially induce some membrane damage and the generation of hydrogen peroxide (H_2O_2) in an oxidative burst, which leads to the activation of defence genes. Different from herbicidal metabolic action injuring plants, the inertness of safeners to plants does not cause toxicity. Hence, the initial decrease in OASTL activity after treatment would be regarded as an unspecific response of the plant to stress. Thereafter, the safening action prevails and the chemicals activate defence responses inducing OASTL activity, leading to enhanced GSH availability (Bartucca et al., 2016).



3. THIOLS PLAY AN IMPORTANT ROLE IN DETOXIFICATION

3.1 Metallothioneins

MTs are cysteine-rich low-molecular-weight (<10 kDa) metal-chelating proteins in many organisms (Fig. 1) (Anjum et al., 2015; Freisinger, 2008). Plant MTs are divided into four groups based on the number and arrangement of their cysteine residues (Cobbett & Goldsbrough, 2002). Type 1–3 MTs contain two cysteine-rich domains connected by a cysteine-poor linker region, the length of which depends on the specific MT type and the plant species (Leszczyszyn, Imam, & Blindauer, 2013). The cysteine-rich α- and β-domains are involved in metal binding. The α-domain is located at the C-terminus of the protein and contains six cysteine residues arranged according to the consensus sequence CxCxxxCxCxxCxC, where x represents any other amino acid besides cysteine. The β-domain is present at the N-terminus and has a more variable amino acid sequence. It generally contains six cysteine residues in MT1

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proteins, eight in MT2 proteins and four in MT3 proteins. Type 4 MTs — also referred to as E_c proteins — can be distinguished from other MT classes based on the presence of three cysteine-rich domains separated by two linker regions. Furthermore, they are characterized by two highly conserved histidine residues in the central cysteine-rich domain (Freisinger, 2008, 2011). In contrast to PCs, which are also important metal chelators in plant cells (see Section 3.2.1), MTs are encoded by genes and thus are products of mRNA translation (Anjum et al., 2015). Although almost all MT genes contain an intron near the N-terminal cysteine-rich domain, it is interesting to note that the exact position of this intron varies according to the specific type of MT encoded (Cobbett & Goldsbrough, 2002).

Different types of MTs display distinct spatiotemporal expression patterns. In general, type 1 MTs are more strongly expressed in roots than in shoots, whereas the opposite holds true for type 2 MTs (Guo, Bundithya, & Goldsbrough, 2003). Although type 3 MTs are also present in leaves, they are mainly expressed in ripening, fleshy fruits (Clendennen & May, 1997; Moyle, Fairbairn, Ripi, Crowe, & Botella, 2005). In contrast, MTs of type 4 are exclusively localized in developing seeds, implying a role in metal storage and accumulation in seeds (Guo et al., 2003). As proposed by Ren et al. (2012), these MTs could provide a means to store Zn required for seed germination. Furthermore, different MT genes are strongly upregulated in ageing leaves, suggesting their involvement in leaf senescence. During this process, MTs possibly serve a dual function. First, they could protect cells against metal toxicity arising from the breakdown of different metal-containing cellular components. Second, they could also be involved in translocating the released metals to nonsenescing plant tissues (Guo et al., 2003; Leszczyszyn et al., 2013).

Although many questions remain with regard to the different functions of plant MTs, their role in metal chelation is well established. MTs have the ability to bind both mono- and divalent metal ions in typical metal-thiolate clusters, which are characterized by high thermodynamic and low kinetic stability. As a consequence, metals are tightly bound by MTs, but part of the metal ions can be readily relocated to other proteins (Hassinen, Tervahauta, Schat, & Karenlampi, 2011). MTs chelate both essential and nonessential metals, indicating their involvement in both nutrient homeostasis and metal detoxification. The metals Zn, Cu and Cd are bound to MTs with the highest affinity (Blindauer & Leszczyszyn, 2010). The importance of MTs in detoxifying excess metals is highlighted by their induction during metal exposure, as discussed in Section 3.1.1. However,

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many other biotic and abiotic stress factors including pathogen attack (Dauch & Jabaji-Hare, 2006), wounding (Razem & Bernards, 2002), light (Chen et al., 2003), drought (Li et al., 2016) and low temperature (Zhu et al., 2009) also induce the expression of *MT* genes, suggesting additional roles besides metal chelation. Transcriptional responses of MTs to different stress factors are possibly mediated by the presence of upstream regulatory elements in the promoter regions of genes encoding these proteins. Metal-, antioxidant-, wounding- and stress-responsive elements were identified in the promoters of type 1, 2 and 3 MTs. Elements responsive to different phytohormones including ethylene, methyl jasmonate, gibberellic acid and salicylic acid were also found in MT promoters, suggesting hormonal regulation of MT expression (Leszczyszyn et al., 2013).

Interestingly, MTs also exhibit antioxidant properties. This is illustrated in *N. tabacum*, where ectopic expression of a type 1 MT from *O. sativa* clearly decreased H_2O_2 accumulation during salinity stress (Kumar et al., 2012). Similar results were obtained by Xue et al. (2009), who reported increased stress tolerance and decreased H_2O_2 levels in transgenic *N. tabacum* plants overexpressing the *Gossypium hirsutum MT3a* gene as compared to the wild type (WT) exposed to cold, salt and drought stress. Furthermore, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) oxidized MT3a in vitro, suggesting the ROS scavenging capacity of this MT (Xue et al., 2009). H_2O_2 levels were significantly higher in an *A. thaliana* T-DNA insertion line lacking functional MT2a as compared to the WT subjected to drought and cold stress (Zhu et al., 2009). Additional evidence supporting an antioxidative function for MTs is provided by the observation that exposure to ROS or ROS-inducing stress factors (e.g., paraquat) increased the expression of several *MT* genes in many plant species including *Quercus suber* (Mir et al., 2004), *G. hirsutum* (Xue et al., 2009), *Ipomoea batatas* (Kim, Jeong, Ahn, Lee, & Kwak, 2014) and *O. sativa* (J. Liu et al., 2015). The antioxidative function of MTs can be explained by the fact that the cysteine residues responsible for metal scavenging also have the capability to reduce ROS. During metal exposure, MTs could also exert an indirect antioxidative function by chelating redox-active metals such as Cu, thereby preventing ROS formation as a consequence of the Fenton and Haber–Weiss reactions (Hassinen et al., 2011; Leszczyszyn et al., 2013).

3.1.1 Responses to Metal Stress

As mentioned earlier, metal exposure induces MT expression in numerous plant species. Exposure to Cu, for example, induced the expression

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of *MT1a*, *MT2a*, *MT2b* and *MT3* in different organs of *A. thaliana*. This increased transcription was especially pronounced in leaf trichomes, which are often reported to accumulate large amounts of metal ions (Guo et al., 2003). Similarly, Cd exposure significantly induced the expression of *MT2a* in *A. thaliana* roots and leaves (Jozefczak et al., 2014). In another study, 24 h exposure to Cd, Cu or Zn was shown to induce the expression of *MT1* in *Cajanus cajan* (Sekhar, Priyanka, Reddy, & Rao, 2011). These metals also increased *MT3* transcription levels in leaves of *Porteresia coarctata* (Usha, Keeran, Harikrishnan, Kavitha, & Parida, 2011). In addition to Cd, Cu and Zn, which are most often associated with MT binding, other metals were shown to induce MT expression as well. Exposure to Pb, for example, strongly induced *MT2a* and *MT2b* expression in shoots of *Hirschfeldia incana* (Auguy et al., 2016). Similarly, *MT1* and *MT2* expression levels were elevated in As-exposed *O. sativa* (Nath et al., 2014). Furthermore, Fe (Ahn et al., 2012), Mn (Ahn et al., 2012; Zhao et al., 2012), mercury (Hg) (Venkatachalam, Srivastava, Raghothama, & Sahi, 2009) and Cr (Gautam et al., 2012) were shown to induce the expression of different *MT* genes in a broad range of plant species. The induction of MT expression in metal-exposed plants suggests a role for these proteins in metal stress defence responses.

A positive role for MTs in metal tolerance is also suggested by several studies reporting differences in *MT* expression between metal-sensitive and metal-tolerant plants. Indeed, *MT3* transcript levels were approximately 2.5-fold higher in the metal hyperaccumulator *Thlaspi caerulescens* than in the nonaccumulator *A. thaliana* grown under control conditions. The cysteine positions in the MT amino acid sequence of both species were different, predicting a smaller metal binding cavity in *A. thaliana* than in *T. caerulescens* (Roosens, Bernard, Leplae, & Verbruggen, 2004). Interestingly, *MT2a*, *MT2b* and *MT3* expression levels were also 1.5- to 4-fold higher in the metal hyperaccumulator *A. halleri* than in *A. thaliana* (Chiang, Lo, & Yeh, 2006). In addition, *MT2b* expression was considerably higher in two independently evolved Cu-tolerant *Silene paradoxa* populations than in a Cu-sensitive population, both under control conditions and after Cu exposure. This effect is probably related to the presence of multiple *MT2b* gene copies in both Cu-tolerant populations (Mengoni et al., 2003). Hassinen et al. (2009) reported that *MT2* and *MT3* were more highly expressed in metallicolous *T. caerulescens* accessions than in a nonmetallicolous accession. However, Zn accumulation and *MT* transcript levels did not cosegregate, implying that MTs are not the major determinants of Zn accumulation in these plants (Hassinen et al., 2009).

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A role for MTs in plant defence against metal stress is also supported by the fact that plants overexpressing different *MT* genes often display enhanced metal tolerance. Overexpression of a putative MT from *Colocasia esculenta* (*CeMT*), for example, reduced the negative effects of Cd, Cu and Zn exposure on root growth in *N. tabacum* seedlings. Furthermore, it significantly increased metal accumulation in these plants. The positive function of this MT during metal stress could be related not only to its metal-chelating function but also to its antioxidant properties. This is confirmed by significantly reduced H_2O_2 and lipid peroxidation levels after 24 h of metal exposure in the *CeMT*-overexpressing plants as compared to their WT counterparts (Kim, Jung, Kim, & Bae, 2013). Similarly, overexpression of *MT2* from *S. alfredii* resulted in increased Cd and Zn tolerance and accumulation in *N. tabacum*. Again, this response was accompanied by significantly decreased H_2O_2 levels in metal-exposed transgenic tobacco plants as compared to the WT. When exposed to Cd and Zn, the *MT2*-overexpressing plants also displayed increased superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) activities in comparison to WT plants (J. Zhang et al., 2014). Overexpression of MTs derived from yeast and mammals can also increase metal tolerance and/or accumulation in plants (Daghan, Arslan, Uygur, & Koleli, 2013; Ruiz, Alvarez, Torres, Roman, & Daniell, 2011; Vrbova et al., 2013) and is proposed to be an interesting phytoremediation strategy.

Although MTs are important players in plant metal stress responses, it should be noted that their responses depend on many factors, including the plant species, tissue and specific metal considered. Schiller et al. (2014), for example, demonstrated that leaf expression levels of *MT1a* were upregulated by Cu and Zn exposure and downregulated by Cd exposure in *Hordeum vulgare*. Similarly, transcript levels of *MT2c* and *MT3* increased in response to Cu, whereas they decreased in leaves of Cd-exposed plants. In contrast, expression levels of these *MTs* remained unchanged in response to Zn. Furthermore — as mentioned earlier — expression levels of different MTs were strongly tissue-dependent, with *MT1b* and *MT4* transcripts exclusively detected in roots and grains, respectively (Schiller et al., 2014). Taken together, these data point towards distinct physiological functions for different MT isoforms. The first step towards unravelling these functions involves the analysis of MTs at the protein level. This remains a challenge, however, as plant MTs are highly prone to proteolysis, hampering the application of proteomics approaches (Blindauer & Leszczyszyn, 2010).

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3.2 Glutathione

GSH is the most abundant nonprotein thiol in almost all aerobic species, occurring at intracellular concentrations of 0.5–10 mM. It is a tripeptide consisting of γ-glutamate, cysteine and glycine and is synthesized in two ATP-dependent steps (Fig. 1). First, a peptide bond is formed between γ-glutamate and cysteine, producing γ-glutamylcysteine (γ-EC). This reaction is catalyzed by γ-EC synthetase (γ-ECS; GSH1), also known as glutamate–cysteine ligase (GCL), and constitutes the rate-limiting step in GSH biosynthesis. Subsequently, GSH is formed by the addition of glycine to γ-EC in a reaction catalyzed by glutathione synthetase (GSH-S; GSH2) (Noctor et al., 2012). GSH1 is exclusively localized in chloroplasts, whereas GSH2 is also present in the cytosol, with cytosolic GSH2 activity strongly exceeding that in chloroplasts (Wachter, Wolf, Steininger, Bogs, & Rausch, 2005). Transport of γ-EC into the cytosol, necessary for cytosolic GSH production, is mediated by chloroquine-resistance transporter-like transporters (CLTs) in the plastid envelope (Maughan et al., 2010). Once synthesized, GSH is distributed to different organelles by the action of plastidial CLTs and transporters in mitochondrial and nuclear membranes (Zechmann, 2014). Mitochondria, chloroplasts and the cytosol have the highest GSH abundance in plant cells (Noctor et al., 2012). In addition to its intracellular transport, GSH is also transported between cells and is one of the major reduced sulphur forms translocated in the phloem (Noctor et al., 2012).

An important determinant of GSH biosynthesis is GSH1 activity, which is regulated at three levels. First, GSH1 activity is subject to feedback inhibition by GSH itself. Under stress conditions, GSH is consumed and the feedback inhibition is alleviated, thereby increasing GSH synthesis. In addition, GSH1 is transcriptionally regulated via the interaction of a redox-sensitive repressor-binding protein with the 5′-untranslated region of its encoding gene (Noctor, Gomez, Vanacker, & Foyer, 2002). Furthermore, GSH1 is posttranscriptionally modulated by the cellular redox state. Under oxidizing conditions, it functions as a homodimeric enzyme with two intermolecular disulphide bonds. Reducing conditions, however, disrupt one of these disulphide bonds, thereby altering the dimer interface and shifting the enzyme to its less active monomeric form (Hothorn et al., 2006). Whether this mechanism is responsible for GSH1 feedback inhibition by GSH is currently unknown (Jozefczak, Remans, Vangronsveld, & Cuypers, 2012). It is interesting to note that GSH biosynthesis is tightly

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coupled to sulphate assimilation, as stress-induced GSH accumulation is often accompanied by increased sulphate uptake and transcriptional upregulation of sulphate assimilation genes, including *APR* and *SAT* (Noctor et al., 2012; Queval et al., 2009; Smith, Kendall, Keys, Turner, & Lea, 1985).

In plants, GSH is involved in many processes including cell cycle regulation and defence against biotic and abiotic stress factors (Noctor et al., 2012). The essential role of GSH in plant physiology is underlined by the observation that plants lacking GSH due to a knockout mutation in either *GSH1* or *GSH2* display a lethal phenotype (Cairns, Pasternak, Wachter, Cobbett, & Meyer, 2006; Lim, Meyer, & Cobbett, 2011). In contrast, plants with lower GSH levels are viable but show an altered phenotype as compared to WT plants. For example, the *root meristemless 1-1* (*rml1-1*) mutant contains less than 5% of WT GSH levels and thereby fails to develop a root apical meristem (Cheng, Seeley, & Sung, 1995; Vernoux et al., 2000). Other GSH-deficient mutants such as *cadmium-sensitive 2-1* (*cad2-1*), *regulator of ascorbate peroxidase 1-1* (*rax1-1*) and *phytoalexin-deficient 2-1* (*pad2-1*) display GSH levels between 25% and 50% of WT levels. Even though these plants develop normally, they are more sensitive to environmental stress factors, emphasizing the crucial role of GSH during stress (Ball et al., 2004; Howden, Andersen, Goldsbrough, & Cobbett, 1995; Parisy et al., 2007).

In this review, we will focus on the most important functions of GSH in stress responses induced by metals and organic contaminants. These include (1) metal chelation by PCs, (2) detoxification of xenobiotics by glutathione transferases (GSTs) and (3) antioxidative defence (Fig. 1).

3.2.1 Glutathione in Chelation: Phytochelatins

An important role of GSH during stress relies on its function as a precursor of PCs. These molecules are important metal scavengers in plant cells and are characterized by a typical $(\gamma\text{-Glu-Cys})_n$-Gly structure, with n ranging from 2 to 11 (Zagorchev, Seal, Kranner, & Odjakova, 2013). PCs are synthesized by PC synthase (PCS) in a two-step reaction. In the first step, the cysteine–glycine peptide bond of a donor GSH molecule is cleaved, yielding γ-EC and glycine. Subsequently the γ-EC unit is transferred to an acceptor molecule – either another GSH molecule or an oligomeric PC peptide – in a transpeptidation reaction. Even though its encoding gene is constitutively expressed, PCS activity is dependent on the presence

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of metals. Although Cd is the most important inducer of PCS activity, other metal(loid)s including As, Cu, Hg, Ni, Pb and Zn were also reported to increase PC synthesis (Anjum et al., 2014). The metal-induced activation of PCS is related to the presence of four highly conserved cysteine residues in its N-terminal domain, which also contains the active site. The C-terminal domain, in contrast, is rich in cysteine residues that possibly bind metal ions to transfer them to the N-terminal active site (Vestergaard et al., 2008). Interestingly, Cd–PC complexes were demonstrated to be translocated into the vacuole, possibly reducing Cd-induced damage to other cellular compartments (Cobbett & Goldsbrough, 2002). In *A. thaliana*, this vacuolar translocation is likely mediated by the ATP-binding cassette (ABC) C3 (ABCC3) transporter, which is transcriptionally induced by Cd exposure (Brunetti et al., 2015).

As mentioned earlier, PC synthesis is induced by several metals. The importance of PCs in metal-induced plant responses is further supported by the fact that the PC-deficient *A. thaliana* mutants *cad1-3* and *cad1-6* – carrying a mutation in the *PCS1* gene – display an enhanced sensitivity towards several metals such as Cd, Pb and Zn (Fischer, Kuhnlenz, Thieme, Schmidt, & Clemens, 2014; Tennstedt, Peisker, Bottcher, Trampczynska, & Clemens, 2009). Furthermore, differences in the extent of PC synthesis between metal-sensitive and metal-tolerant varieties are often observed. Indeed, a Cd-tolerant *Triticum aestivum* cultivar displayed stronger Cd-induced increases in GSH levels and *PCS* expression in comparison to a Cd-sensitive cultivar (Kumari, Parmar, & Sharma, 2015). Similarly, *PCS1* expression and total PC levels were significantly increased by As exposure in an As-tolerant *O. sativa* cultivar, whereas they remained unaffected in its As-sensitive counterpart. As-induced increases in GSH, cysteine and methionine levels were also more pronounced in the tolerant cultivar than in the sensitive cultivar (Begum et al., 2016).

As PCs are efficient metal chelators, transgenic plants with increased PC levels are often able to accumulate high metal concentrations. For example, *N. tabacum* plants overexpressing *PCS1* displayed an enhanced accumulation of Cd, Cu, Ni and Zn as compared to WT plants. These plants even accumulated higher metal concentrations than the metal hyperaccumulator *T. caerulescens* (Martínez et al., 2006). The use of transgenic plants with increased PC production could therefore offer a promising strategy for the phytoremediation of metal-polluted soils. However, it should be noted that overproduction of PCs is not always beneficial for plants, as it can cause a strong decrease in cellular GSH levels. Since GSH also serves important

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antioxidative functions (see Section 3.2.3), GSH depletion possibly results in oxidative stress and cellular damage. This was shown by Jozefczak et al. (2014), who reported a rapid increase in PC levels in roots and leaves of *A. thaliana* plants exposed to 5 and 10 μM Cd. In the roots, this response coincided with a fast (2 h) reduction in GSH levels. Despite the activation of alternative antioxidative defence systems after 24 h, decreased biomass and increased lipid peroxidation were observed in Cd-exposed plants (Jozefczak et al., 2014). These results indicate that plant metal tolerance is related not only to the production of PCs but also to the ability to prevent the resulting GSH depletion. Therefore, the use of transgenic plants overexpressing both *PCS* and GSH biosynthesis genes could be more efficient to remediate metal-contaminated areas (Seth et al., 2012).

3.2.2 Glutathione in Detoxification: Glutathione Transferases

Another important function of GSH is related to its involvement in detoxification reactions mediated by GSTs (Fig. 1). These enzymes catalyze the conjugation of xenobiotics to GSH by addition or substitution reactions to reduce their toxicity and increase their water solubility (Coleman, Blake-Kalff, & Davies, 1997; Dixon et al., 2010). As GSTs possess broad and overlapping substrate specificities, they are able to detoxify a chemically diverse array of compounds. Xenobiotics susceptible to GSH conjugation include electrophilic herbicides, several drugs and organic contaminants (Cole, Cummins, Hatton, Dixon, & Edwards, 1997; Frova, 2003). Generally, these compounds are characterized by the presence of two carbon atoms coupled by a double bond, adjacent to an electron-withdrawing group (Frova, 2003). Given the reactivity of these molecules, conjugation to GSH occurs spontaneously but is considerably speeded up by GST activity (Coleman et al., 1997). In addition to their role in GSH conjugation reactions, GSTs also serve a broad range of other catalytic as well as noncatalytic functions (Frova, 2003). Although the molecular structure and catalytic mechanisms of GSTs have been studied by several research groups, their natural functions remains largely obscure. An overview of the natural GST functions elucidated so far is provided in Table 1. The first functions ascribed to GSTs were all related to conjugation (e.g., of xenobiotics), whereas more sophisticated functions such as the synthesis of glucosinolates were only discovered more recently (Table 1).

Even though most GSTs are soluble proteins present in the cytosol, they have also been detected in several organelles including chloroplasts, mitochondria, peroxisomes, the vacuole and nucleus.

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Table 1 Natural Functions of Glutathione Transferases (GSTs) in Plants

Postulated Natural Role of GSTs	References
Conjugation of endogenous metabolites	Diesperger and Sandermann (1979), Edwards and Dixon (1991), and Dean, Devarenne, Lee, and Orlofsky (1995)
Conjugation of DNA degradation products	Morgenstern, Depierre, and Jornvall, (1985)
Conjugation of phytohormones (auxin, ethylene, gibberellic acid)	Lamoureux and Frear (1987), Meyer, Goldsbrough, and Woodson (1991), Takahashi and Nagata (1992), and Zettl, Schell, & Palme (1994)
Detoxification of lipid hydroperoxides	Mannervik and Danielson (1988), Marrs (1996), and Sommer and Boger (1999)
Transport of (thio-)phenols, chlorophyllin and anthocyans	Singh and Shaw (1988) and Martinoia, Grill, Tommasini, Kreuz, and Amrhein (1993)
Regulation of GSH pool	Lamoureux and Rusness (1989)
Detoxification of fungal toxins and pathogen defence	Dudler, Hertig, Rebmann, Bull, and Mauch (1991) and Mauch and Dudler (1993)
Increase of drought tolerance	Dhindsa (1991)
Antioxidative protective protein	Levine, Tenhaken, Dixon, and Lamb (1994)
Conjugation and transport of phytoalexins and similar compounds	Li, Alfenito, Rea, Walbot, and Dixon (1997) and Marrs, Alfenito, Lloyd, and Walbot (1995)
Regulation of UV-induced genes	Loyall, Uchida, Braun, Furuya, and Frohnmeyer (2000)
Transporter proteins for secondary metabolites and their unstable intermediates	Dixon et al., 2010
Synthesis of glucosinolates; conjugation, transport and storage of reactive oxylipins, phenolics and flavonoids	Dixon et al. (2010) and Dixon and Edwards (2010)
Isomerization and peroxidation	Cummins, Dixon, Freitag-Pohl, Skipsey, and Edwards (2011)
Deglutathionation of GS-conjugates	Lallement et al. (2014)

Microsomal GSTs belonging to the 'membrane-associated proteins in eicosanoid and glutathione metabolism' (MAPEG) family have also been characterized (Oztetik, 2008). For example, microsomal GSTs with herbicide-detoxifying activities have been detected in *Pachyrhizus erosus*

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(Belford, Dorfler, Stampfl, & Schroder, 2004) and in lower plants (Pflugmacher, Schroder, & Sandermann, 2000). It is important to note that cytosolic GSTs are very abundant, constituting up to 2% of all soluble proteins in plants (Scalla & Roulet, 2002).

To date, plant GSTs are subdivided into several classes according to a classification system proposed by Droog (1997) and subsequently refined by Edwards, Dixon, and Walbot (2000). Using this system, GSTs are classified based on gene organization (number and position of introns), amino acid similarity and conservation of specific residues in the protein. The phi (GSTF) and tau (GSTU) classes constitute the largest classes of plant GSTs and are plant specific. Interestingly, they are also predominantly active in xenobiotic detoxification (Dixon et al., 2010; Frova, 2006). Indeed, phi class GSTs have been cocrystallized with a range of herbicide and other xenobiotic conjugates (Prade, Huber, & Bieseler, 1998). In contrast, the zeta (GSTZ) and theta (GSTT) families are much smaller and are related to mammalian GSTs. Whereas GSTs belonging to these classes display GSH-conjugating activities, other classes including the lambda GSTs (GSTL) and dehydroascorbate reductases (DHARs) have no known ability to conjugate or detoxify synthetic compounds. Instead, they seem to have a redox-related role (Dixon, Davis, & Edwards, 2002).

Structurally, most cytosolic GSTs occur as dimers consisting of two subunits (approximately 25 kDa each), each encoded by one gene (Oztetik, 2008). Although homodimers and heterodimers can be formed, dimerization has only been observed between GSTs from the same class (Armstrong, 1997). An exception are the DHARs and lambda GSTs, which function as monomers (Dixon et al., 2010). Each GST subunit is characterized by a common overall structure with a well-defined N-terminal GSH-binding domain (G-site) and a less specific C-terminal domain binding the hydrophobic cosubstrate (H-site). Most GST classes, including the phi, tau, zeta and theta class, contain a serine in the active site responsible for the formation and stabilization of the reactive thiolate anion from GSH, which is the target for the nucleophilic attack of an electrophilic substrate (Dixon et al., 2010). In DHARs and lambda GSTs, however, the serine is replaced by a cysteine that forms a mixed disulphide with GSH. The catalytic cysteine performs a nucleophilic attack on GSH-conjugated substrates. Under these conditions, the catalytic cysteine of the GST becomes glutathionylated, thereby deconjugating the substrate that is subsequently released. Regeneration of the resulting glutathionylated GST forms requires a GSH molecule, forming GSSG as another end product. The

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reduced GSTs can be used in another catalytic cycle and GSSG is reduced back to GSH by glutathione reductase (GR) at the expense of NADPH (Lallement, Brouwer, Keech, Hecker, & Rouhier, 2014).

In general, phi and tau GST-catalyzed substitution reactions change the parent compound to an extent that the toxicity of the xenobiotic is significantly lowered (Schroder & Collins, 2002). Along with the resulting increase in polarity, GSH addition is a very effective means of detoxification, especially when the electrophilic centre of the target molecule is a leaving group. In some situations, however, the formation of glutathione S (GS)-conjugates could be disadvantageous to plants when they accumulate in high amounts. In *Picea abies*, for example, it is clearly proven that GS-conjugates accumulating in the cytosol inhibit the stress-reducing activity of GR (Schroder & Pflugmacher, 1996). Furthermore, when detoxification reactions withdraw GSH from the cellular pool, GSH depletion due to exaggerated utilization in conjugation reactions or inhibition of GSH supply could become a problem, particularly when the xenobiotic conjugates still remain reactive and/or are not efficiently stabilized (Brazier-Hicks et al., 2008). However, GS-conjugate formation has generally been accepted to be beneficial to plants (Edwards et al., 2000; Schröder, 2006).

Furthermore, conjugation of substrates with GSH allows them to be transported into the vacuole by ABC transporters (Mohanasundaram et al., 2015; Theodoulou, 2000). This removal of GS-conjugates from the cytosol is of ample importance, as they can inhibit the activities of GSTs as well as other GSH-dependent enzymes (cfr. *supra*) (Coleman et al., 1997). Once present in the vacuole, GS-conjugates can undergo further metabolism, as discussed by Coleman et al. (1997).

Interestingly, GSTs are induced by a broad range of biotic and abiotic stress conditions, including exposure to organic contaminants. The relevance of this induction is mostly related to direct detoxification of xenobiotics, but can also be explained by the fact that certain GSTs detoxify products of lipid peroxidation and oxidative DNA damage. This characteristic of GSTs also possibly underlies their involvement in plant responses to metal stress (Frova, 2003).

3.2.2.1 Responses to Stress Conditions

As mentioned earlier, GSTs are important players in plant responses to organic compounds. In this context, especially herbicides are known to affect plant GST levels and activities. For example, glyphosate significantly increased GST activities in *Pisum sativum* roots and leaves (Miteva, Ivanov,

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& Alexieva, 2010). Similarly, exposure to the chloroacetamide herbicide metazachlor induced GST activity in *B. napus* leaves (Vercampt, Koleva, Vassilev, Vangronsveld, & Cuypers, 2016). The role of GSTs in herbicide detoxification is further supported by the enhanced tolerance to the diphenyl ether herbicide fluorodifen of *N. tabacum* plants overexpressing a *Citrus sinensis* tau class GST. Interestingly, salt and drought tolerance were also increased in the overexpressor plants. However, this effect was not due to scavenging of oxidative stress by-products, suggesting additional GST-mediated mechanisms provoking tolerance to these stress conditions (Lo Cicero, Madesis, Tsaftaris, & Lo Piero, 2015). Even though GSTs play an important role in detoxifying herbicides, their activities are not always induced in herbicide-exposed plants. The photosynthetic herbicide isoproturon reduced GST activity in *Z. mays* leaves, indicating that certain herbicides function as GST inhibitors (Alla, Hassan, El-Bastawisy, 2008).

Herbicide safeners often exert their function by increasing the rate of GSH conjugation in several crop species (Abu-Qare & Duncan, 2002; Davies & Caseley, 1999). For example, the safener benoxacor induced the activity of a tau class GST in *Festuca arundinacea* (Del Buono, Scarponi, & Espen, 2007). Furthermore, the safener cloquintocet-mexyl was shown to increase the protein abundance of several GSTs belonging to the phi, tau and lambda classes in the roots and coleoptile of *Triticum tauschii* seedlings (Zhang, Xu, Lambert, & Riechers, 2007). However, it should be noted that safeners could also increase the GSH conjugation rate in weeds competing with the target crop. Mefenpyr-diethyl and fenchlorazole-ethyl safeners, for example, were shown to increase the protein abundance of certain phi and lambda class GSTs in the weed *Alopecurus myosuroides* treated with the graminicidal herbicide fenoxaprop-ethyl (Cummins, Bryant, & Edwards, 2009). In addition, in *A. thaliana* seedlings, the gene encoding GSTU26 was transcriptionally induced by exposure to the chloroacetanilide herbicides alachlor and metolachlor and the safener benoxacor. In contrast, expression levels of *GSTF9* were not affected under these conditions, indicating that specific GST isoforms are responsive to specific safeners and/or herbicides (Nutricati, Miceli, Blando, & De Bellis, 2006). Similarly, different safeners were shown to transcriptionally induce several phi and theta class GSTs in *A. thaliana*. Nonetheless, this response did not protect the plants from herbicide-induced damage, suggesting that other players besides GSTs are responsible for the differences in safener-induced herbicide tolerance between plant species (DeRidder, Dixon, Beussman, Edwards, & Goldsbrough, 2002).

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Furthermore, it should be noted that GSTs can bioactivate instead of detoxify certain organic compounds. Two examples of toxic GS-conjugates in plants refer to GST-mediated isomerization involved in herbicide bioactivation of isourazoles and thiadiazolidines to toxic urazoles and triazolidines, respectively (Edwards et al., 2000).

In addition to herbicides and safeners, metal exposure is also known to affect GSTs in plants. As mentioned earlier, this observation is likely related to the involvement of GSTs in detoxifying products resulting from oxidative damage to cellular macromolecules. Exposure to Cd, Cu, cobalt (Co), Hg, Ni and Zn significantly increased GST activity in root tips of *H. vulgare*. This response was also induced by salt, cold, drought and H_2O_2 exposure (Halušková, Valentovičová, Huttová, Mistrík, & Tamás, 2009). Furthermore, Cd was shown to induce GST activity in the roots and leaves of *Ricinus communis*. This Cd-induced increase in GST activity was also observed in *B. napus* roots. As Cd exposure significantly increased MDA concentrations in both plant species, the induction of GST activity could be targeted towards detoxifying lipid peroxidation products (Bauddh, Kumar, Srivastava, Singh, & Tripathi, 2016). Kumar et al. (2013) reported that gene expression levels of *GSTL1* and *GSTL2* were increased by As, Cd and Pb exposure in *O. sativa* seedlings, whereas they were not affected by Cr. *GSTL3* expression was induced by As and Pb, but not by Cd. Taken together, these data indicate that metal-induced transcriptional responses of GSTs depend on the metal and GST isoform under study (Kumar et al., 2013; Lyubenova & Schroder, 2011).

3.2.3 Glutathione in Antioxidative Defence

GSH, as the most abundant essential nonenzymatic antioxidant in plant cells, contributes to antioxidative defence in several ways. First, it can directly react with singlet oxygen ($^1O_2$), H_2O_2 and hydroxyl radicals ($^{\bullet}OH$) (Gill & Tuteja, 2010b). In addition, GSH plays an important role in the antioxidative ascorbate (AsA)-GSH cycle, also referred to as the Foyer-Halliwell-Asada pathway. In this cycle, H_2O_2 is reduced to H_2O by the action of ascorbate peroxidase (APX), simultaneously oxidizing AsA to dehydroascorbate (DHA). The latter molecule is again reduced to AsA by the action of DHAR, using GSH as an electron donor (Fig. 1) (Foyer & Noctor, 2011). GSH also contributes to antioxidative defence as an electron donor for glutathione peroxidase (GPX), catalyzing the reduction of H_2O_2, organic peroxides and lipid peroxides (Anjum et al., 2012; Gill & Tuteja, 2010b). In addition, it reduces glutaredoxins (GRXs), which are involved

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in several processes including the regeneration of antioxidative enzymes (Rouhier, 2010).

In all the reactions described earlier, GSH donates an electron – derived from the cysteine residue of its thiol group – to another molecule and thereby becomes reactive. It then readily reacts with another reactive GSH molecule, forming glutathione disulphide (GSSG). Subsequently, GSSG can be reduced to GSH by the action of the NADPH-dependent GR enzyme (Fig. 1). Plants possess two GR-encoding genes: (1) *GR1*, present in the cytosol and (2) *GR2*, dually targeted to plastids and mitochondria (Noctor et al., 2012). As GR is constitutively active, more than 90% of the cellular GSH pool is in its reduced form under control conditions. During stress conditions, however, GSH demand can exceed GR activity, thereby decreasing the GSH/GSSG ratio (Jozefczak et al., 2012). As the most abundant redox couple in plant cells, GSH/GSSG plays a crucial role in redox regulation (Noctor et al., 2012). However, the relevance of the GSH/GSSG redox potential as the driving force of biological processes has to be critically discussed (Flohe, 2013).

Plant GSH levels and redox state are often affected by metal(loid) exposure (Jozefczak et al., 2012). For example, Dixit et al. (2016) demonstrated that exposure to 25 and 50 μM as significantly increased GSH and GSSG levels in both roots and shoots of *O. sativa* plants. The observed oxidation of GSH was related to the activation of the AsA-GSH cycle, as indicated by significantly increased APX activities in both organs. However, the GSH/GSSG ratio remained unaffected after As exposure. This is probably due to the observed increases in the activities of GR and enzymes involved in sulphate assimilation and GSH biosynthesis, such as SAT and GSH1. It is interesting to note that under all conditions, GSH levels were positively correlated with sulphur concentrations in the growth medium, whereas GSSG levels decreased with increasing sulphur levels. These data indicate that antioxidative defence in As-exposed plants is more efficient under high-sulphur conditions (Dixit et al., 2016). Furthermore, the GSH redox state was significantly shifted towards a more reduced state in leaves of *A. thaliana* exposed to Zn concentrations ranging from 100 to 500 μM, despite a significant decrease in GR activity. However, the increased GSH/GSSG ratio could be explained by an increased GSH synthesis, as Zn significantly increased total GSH equivalents and free GSH levels. In contrast, Zn slightly decreased the GSH/GSSG ratio in roots, possibly as a result of the observed decrease in root GR activity. PC synthesis was strongly induced, thereby

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decreasing free GSH levels available for antioxidative defence reactions in roots of Zn-exposed plants (Remans et al., 2012). Cuypers et al. (2011) reported that both Cd and Cu exposure shifted the GSH redox state in *A. thaliana* roots towards a more oxidized state, which was much more pronounced in Cu-exposed plants. In contrast, the GSH/GSSG ratio was not significantly affected by either metal in the leaves (Cuypers et al., 2011). Nocito et al. (2006) reported that Cd exposure significantly decreased total GSH levels in roots of *Z. mays*. Reduced GSH levels strongly diminished, whereas GSSG concentrations significantly increased in roots of Cd-exposed plants, thereby shifting the cellular redox balance towards a more oxidized state. Zn exposure, in contrast, did not alter the GSH/GSSG ratio, as it increased both GSH and GSSG levels to the same extent. Again, exposure to Cu caused the most dramatic effects, as it decreased the GSH/GSSG ratio by more than 20-fold (Nocito et al., 2006). These data indicate that metal-induced effects related to the GSH/GSSG ratio depend on many factors including the chemical properties of the metal, its concentration, the exposure duration and the plant species and organ considered.

As mentioned earlier, the role of GSH in plants exposed to organic contaminants is mainly related to its involvement in GST-mediated xenobiotic detoxification. In addition, GSH possibly functions in antioxidative defence reactions triggered by organic compounds such as herbicides. Exposure to metazachlor for 14 days increased the activities of APX and GR in leaves of *B. napus*, suggesting activation of the AsA-GSH cycle. This assumption was further supported by the observation that the GSH redox state was significantly shifted towards its oxidized form. Activities of other antioxidative enzymes, including SOD and CAT, were also induced by exposure to metazachlor (Vercampt et al., 2016). Similarly, exposure to the recommended field dose of metribuzin increased GSSG levels and slightly decreased the GSH/GSSG ratio in leaves of *Z. mays*, possibly as a result of the observed decrease in GR activity. In contrast, treatment with the recommended field dose of pretilachlor increased GR activity and the GSH/GSSG ratio. These results possibly explain the fact that the induction of H_2O_2 levels and lipid peroxidation was more pronounced in plants exposed to metribuzin than in those exposed to pretilachlor. Taken together, these data indicate that herbicide-induced antioxidative plant responses depend on the specific herbicide applied (Alla, Badawi, Hassan, El-Bastawisy, & Badran, 2008).

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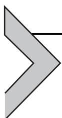

4. THIOLS PLAY AN IMPORTANT ROLE IN CELLULAR REGULATION

4.1 Methionine and S-adenosylmethionine Synthesis

In addition to its function as a building block of proteins and, in particular, MTs, GSH and PCs, cysteine also plays an important role in the biosynthesis of the second sulphur-containing amino acid methionine. Like lysine and threonine, methionine belongs to the family of aspartate-derived amino acids (Hesse et al., 2001). It is composed of (1) a sulphur atom derived from cysteine, (2) the nitrogen/carbon skeleton from a phosphorylated serine and (3) a folate-derived methyl group (Wirtz & Droux, 2005). The synthesis of methionine comprises three steps (Fig. 1). In the first step, cystathionine γ-synthase (CGS) catalyzes the formation of the thioether cystathionine from its substrates cysteine and O-homophosphoserine (OPH). Subsequently, cystathionine β-lyase (CBL) cleaves cystathionine to homocysteine in a β-cleavage reaction. These two steps exclusively take place in plastids and are together referred to as the transsulphuration pathway (Droux, 2004; Wirtz & Droux, 2005). In the third and final step of methionine synthesis, methionine is formed by the methylation of homocysteine, which is catalyzed by a vitamin B12-independent methionine synthase (MS), using methyltetrahydrofolate as the methyl donor (Fig. 1) (Takahashi et al., 2011). This enzyme is present in both plastids and the cytosol. The plastidial MS isoform is involved in only de novo methionine synthesis, whereas the cytosolic isoform also mediates the recycling of homocysteine resulting from the hydrolysis of S-adenosylhomocysteine (Gigolashvili & Kopriva, 2014; Wirtz & Droux, 2005).

More than 80% of the synthesized methionine is used for the production of SAM (Ravanel, Gakiere, Job, & Douce, 1998). This metabolite is produced from methionine and ATP in a cytosolic reaction catalyzed by SAM synthetase (SAMS) (Fig. 1). SAM serves as the methyl donor in a broad array of methylation reactions catalyzed by methyltransferases. Furthermore, SAM is also the precursor for the synthesis of ethylene, polyamines (PAs), nicotianamine, phytosiderophores and biotin (Roje, 2006).

The regulation of methionine and SAM synthesis mainly takes place at the level of the CGS enzyme. Although CGS is not subject to feedback inhibition by methionine or its metabolites at the activity level, it is posttranscriptionally regulated by SAM (Amir, 2010). When SAM is present, it

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induces a temporary arrest of the translation elongation process of the methionine overaccumulation 1 (MTO1) domain in the CGS mRNA, causing degradation of the mRNA upstream of the stalled ribosome. As a result, 5′-truncated RNA species are produced, causing the decay of the transcript (Amir, 2010; Onouchi et al., 2005). The importance of the tight regulation of CGS is highlighted by the observation that plants with increased or decreased CGS levels display severe morphological phenotypes (Amir, 2010).

Control of methionine production also occurs at the level of OPH, which is the last common intermediate for methionine and threonine biosynthesis. Although both CGS and threonine synthase (TS) use OPH as a substrate, the affinity of TS for OPH strongly exceeds that of CGS. In addition, SAM enhances the activity and substrate affinity of TS. Therefore, OPH mainly flows to the threonine synthesis pathway when methionine and SAM levels are sufficiently high. When SAM levels decline, however, TS activity decreases and OPH supply to the methionine synthesis pathway increases (Takahashi et al., 2011).

In addition to cysteine, methionine levels can be affected in metal-exposed plants. Exposure to 120 μM Ni was demonstrated to increase methionine concentrations in roots and shoots of *Matricaria chamomilla* (Kovacik, Klejdus, Hedbavny, & Backor, 2009). Furthermore, exposure to different sublethal Cd concentrations for 24 and 72 h significantly increased methionine concentrations in both roots and leaves of *A. thaliana* (Keunen, Florez-Sarasa, et al., 2016). MS protein abundance was significantly increased by Cd exposure in *T. aestivum* leaves. This response was accompanied by an increased level of 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS), an intermediate in the ethylene biosynthesis pathway (Fig 1). Interestingly, similar effects were induced by exposure to trichlorobenzene, suggesting that both metals and organic contaminants can affect methionine and ethylene biosynthesis pathways in plants (Ge et al., 2009). In contrast, Barkla, Vera-Estrella, Miranda-Vergara, and Pantoja (2014) reported that Zn decreased MS abundance in leaves of *A. thaliana*. Serine hydroxymethyltransferase levels were also reduced in Zn-exposed plants. This enzyme catalyzes the conversion of glycine to serine, thereby yielding methyl units that are channelled into the methionine biosynthesis pathway. The Zn-induced decrease of this enzyme possibly results in a decreased methionine concentration. The authors hypothesize that this mechanism protects Zn-stressed cells by attenuating the initiation of protein translation, thereby preventing the synthesis of

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misfolded proteins (Barkla et al., 2014). A Zn-induced decrease in methionine levels was also observed in shoots of *N. tabacum* (Pavlíková et al., 2014). Exposure to As, however, increased methionine concentrations in *O. sativa*. This response was more pronounced in a genotype accumulating higher As levels. A similar response was observed for cysteine and other stress-responsive amino acids such as proline, glycine and glutamate. Clear correlations were reported among the levels of these amino acids, the extent of lipid peroxidation and the activities of antioxidative enzymes, suggesting that their levels are increased as a defence mechanism against As-induced oxidative stress (Dave et al., 2013).

Further evidence supporting a role for methionine in plant responses to metal stress is again derived from comparing metal-tolerant and metal-sensitive species. Begum et al. (2016) reported that As exposure significantly increased methionine levels in a tolerant *O. sativa* genotype, whereas they were not affected in an As-sensitive genotype. Similar results were demonstrated for cysteine and GSH, underlining the importance of sulphur metabolism in plant metal tolerance (Begum et al., 2016). Similarly, Liang et al. (2014) demonstrated that Cd increased root methionine levels in a hyperaccumulating *S. alfredii* ecotype, whereas this response was absent in its nonhyperaccumulating counterpart. Furthermore, *SAMS* expression levels were only significantly enhanced by Cd exposure in roots and shoots of the hyperaccumulator plants. Taken together, these data point towards a role for SAM in mediating Cd tolerance in *S. alfredii* (Liang et al., 2014). MS and SAMS protein abundance were significantly increased by Cu exposure in roots of a metallicolous population of *Agrostis capillaris*, whereas they were not affected in a nonmetallicolous population, further supporting a role for methionine and SAM in plant metal tolerance (Hego et al., 2014).

The effects of metals on methionine and SAM biosynthesis are relatively well studied, whereas information regarding the influence of organic contaminants on these processes is scarce and therefore constitutes an interesting topic for future research. In this regard, evidence has been presented that SAM-methyltransferase is involved in the detoxification of the herbicide fluorodifen in *Picea*. The fluorodifen metabolite 2-nitro-4-trifluoromethyl-thiophenol was rapidly converted to the corresponding 2-nitro-4-trifluoromethyl-thioanisole, a volatile end product of the degradation pathway in needles of *P. abies*, *Picea glauca* and *Picea pungens* (Lamoureux, Rusness, & Schroder, 1993). A similar pathway was demonstrated for the metabolism of pentachloronitrobenzene in *Allium cepa* (Lamoureux & Rusness, 1980).

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4.2 Ethylene: Hormonal Regulation

As mentioned earlier, thiols are important precursors in a large number of cellular processes and regulatory networks. SAM, the activated form of methionine, is a key player in the synthesis of ethylene and PAs. As a precursor of ethylene, SAM is directly linked to hormonal signalling.

In the ethylene biosynthesis pathway, SAM is converted to ACC by the ACS enzyme belonging to the class of pyridoxal phosphate (PLP)-dependent enzymes utilizing vitamin B6 as a cofactor (Fig. 1). This reaction involves the release of 5′-methylthioadenosine (MTA), which is recycled back to methionine in the so-called Yang cycle (Murr & Yang, 1975). The ACS enzyme is located in the cytosol and encoded by a multigene family of 12 members in *Arabidopsis*, of which 8 encode functional proteins (Van de Poel & Van Der Straeten, 2014). Expression of different members of this large gene family is tissue-dependent and single isoforms are specifically involved in distinct physiological or developmental tasks (Tsuchisaka & Theologis, 2004). Furthermore, complex interactions are known to occur between different ACS members (Tsuchisaka et al., 2009).

The second step in ethylene synthesis consists of the oxidation of ACC to ethylene via ACC oxidase (ACO) in the presence of oxygen (Fig. 1) (Kende, 1993). As a member of the superfamily of dioxygenases, ACO requires Fe^{2+} as a cofactor and bicarbonate as an activator (Zhang, Ren, Clifton, & Schofield, 2004). Information on its cellular localization remains unclear: it could be localized in either the cytosol (Chung, Chou, Kuang, Charng, & Yang, 2002; Hudgins, Ralph, Franceschi, & Bohlmann, 2006) or the plasma membrane (Ramassamy, Olmos, Bouzayen, Pech, & Latche, 1998; Rombaldi et al., 1994). Even though ACS is considered the rate-limiting enzyme of ethylene biosynthesis in plants, there are reports indicating that ACO limits ethylene synthesis under certain conditions, e.g., postclimacteric fruit ripening in tomato (Van de Poel & Van Der Straeten, 2014). In plants, ethylene synthesis is regulated by a variety of internal and external signals, often with ACS as the main target (for a review, see Van de Poel and Van Der Straeten (2014) and Keunen, Schellingen, et al. (2016)).

Many reports have shown enhanced ethylene production upon metal exposure in plants, with the extent depending on the metal and its applied concentration. From all inorganic ions, Cd probably causes the strongest induction of ethylene production in plants (Keunen, Schellingen, et al., 2016). While this was shown for several species under short time exposure conditions, longer Cd exposure decreased ethylene concentrations in

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A. thaliana (Carrio-Segui, Garcia-Molina, Sanz, & Penarrubia, 2015). Summing up literature data, an intimate relationship exists between metal stress and ethylene production in plants. More and more reports suggest an implication of ethylene signalling in plant adaptation and tolerance to metal stress (Keunen, Schellingen, et al., 2016; Thao et al., 2015). As discussed in Section 5, extensive cross talk is observed between ethylene and other key players in the plant metal stress response, such as ROS and GSH.

In contrast with the vast amount of data available on the involvement of ethylene in plant metal stress responses, knowledge on its role in plants exposed to organic contaminants is limited. Nonetheless, Kummerová, Zezulka, Váňová, and Fišerová (2012) have demonstrated a significant stimulation of ethylene release in germinating *Z. mays* and *P. sativum* seeds during exposure to the polycyclic aromatic hydrocarbon fluoranthene. Seed germination was significantly inhibited by this compound in both species (Kummerová et al., 2012).

4.3 Polyamines: Hormonelike Signalling Compounds

Next to ethylene synthesis, SAM is also a precursor for the synthesis of PAs, representing a group of low-molecular-weight polycationic amines ubiquitous in all living organisms (J.-H. Liu, Wang, Wu, Gong, & Moriguchi, 2015). In plants, the positively charged PAs are either bound to negatively charged molecules or conjugated to small molecules and proteins, but they also occur as free forms (Walters, 2003). PAs are involved in a large number of cellular functions and regulatory processes, including the stabilization of proteins and other biomolecules, the regulation of cell division, growth and differentiation or senescence as well as general adaptive stress responses (Bouchereau, Aziz, Larher, & Martin-Tanguy, 1999; Groppa & Benavides, 2008; Walters, 2003).

Putrescine (Put), spermidine (Spd) and spermine (Spm) are the three major PAs and are therefore most studied in plants. They can be present in free, soluble conjugated and insoluble bound forms. In plants, PA synthesis is well studied and based on the two precursors L-ornithine and L-arginine from which Put is generated by the catalytic actions of ornithine decarboxylase (ODC) and arginine decarboxylase (ADC). From Put, both Spd and Spm are formed, with a strong connection to SAM (Fig. 1) (Sauter et al., 2013). In the first step, SAM is decarboxylated to decarboxylated SAM (dcSAM) by SAM decarboxylase (SAMDC). This metabolite then provides the aminopropyl group for the conversion of Put into Spd by Spd synthase.

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Spd is then further converted into Spm by Spm synthase, again using dcSAM as an aminopropyl donor (Fig. 1) (J.-H. Liu et al., 2015). From this reaction, MTA is released and directed back into the SAM cycle where SAM is synthesized from methionine by SAMS (Sauter et al., 2013). Apart from de novo synthesis of PAs, it is worth mentioning that catabolism of these molecules takes place, involving both Cu-containing diamine oxidases (CuAOs) and FAD-dependent polyamine oxidases (PAOs) (Cona, Rea, Angelini, Federico, & Tavladoraki, 2006; J.-H. Liu et al., 2015).

The relationship between abiotic stress and altered (mostly elevated) PA levels is well established and has been known for a long time. Nevertheless, their actual function still remains unclear. Although PAs could protect cells against abiotic stress, for example by their antioxidative properties, they could also cause cell damage by the production of H_2O_2 via their catabolism (Minocha, Majumdar, & Minocha, 2014).

As indicated in Fig. 1, SAM represents a direct link between ethylene and PA synthesis, allowing plants to directly switch between both pathways. Interestingly, PAs and ethylene display counteracting functions in plants. Ethylene is known to induce senescence, whereas PAs play the opposite role by decelerating chlorophyll loss and cell membrane degradation and slowing down senescence-induced increases in protease and RNase activity (Pandey, Ranade, Nagar, & Kumar, 2000). This is because of their ability to neutralize acids, their antioxidative properties and their potential to stabilize cell walls and membranes (Gill & Tuteja, 2010a).

Different reports indicate a role for PAs in plant metal stress responses. For example, exposure to Cu and Cd led to an increase in ADC and ODC activity in *T. aestivum* leaves. This effect was stronger under Cd exposure, where Put levels were elevated up to threefold as compared to control levels. Spd concentration was not affected by any of the metals, whereas Spm levels were significantly reduced. Interestingly, externally applied Spm reduced the formation of H_2O_2 and led to a reduction in lipid peroxidation levels as well. A potential antioxidative function of Spm could be concluded from these results, although the actual mechanism of protection is still unclear (Groppa, Tomaro, & Benavides, 2007).

Although it is well established that in plants many organic pollutants cause the formation of ROS, there is only little information available on how the PA metabolism is affected by these xenobiotic compounds. Nevertheless, Burritt (2008) could link phenanthrene exposure to the synthesis of PAs. Phenanthrene is a polycyclic and highly toxic aromatic hydrocarbon

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that is often found in aquatic environments. Whereas data regarding its effect on aquatic macrophytes are scarce, polycyclic aromatic hydrocarbons often induce oxidative stress in plants. The involvement of PAs in the protection of plants against oxidative stress could be shown for *Riccia fluitans* exposed to phenanthrene. Concentrations up to 500 μM led to an induction of oxidative stress to which the plants responded with an increase in PA synthesis linked to elevated ADC and SAMDC activity. Chemical inhibition of these enzymes caused an inhibition of plant recovery, whereas externally applied PAs could reduce the negative effects caused by phenanthrene exposure (Burritt, 2008). Studies suggest PAs as priming agents before all sorts of abiotic stress, including metal exposure (Savvides, Ali, Tester, & Fotopoulos, 2016). This opens the window to more extensive research on the involvement of PAs in plant responses to metals as well as organic pollutants.

5. INTERACTION BETWEEN DETOXIFICATION AND SIGNAL TRANSDUCTION PATHWAYS

As discussed earlier, sulphur-containing metabolites play an important role in plant responses to metal stress. Compounds directly derived from cysteine (e.g., GSH, PCs and MTs) are involved in metal chelation and ROS detoxification, whereas molecules derived from methionine and its primary metabolite SAM (e.g., ethylene and PAs) mainly function in cellular signal transduction. However, different components of the detoxification and signal transduction pathways interact with each other under both physiological and stress conditions, including metal exposure (Fig. 2).

For example, cross talk exists between ethylene and other players involved in plant responses to metal stress, as reviewed by Keunen, Schellingen, et al. (2016). Several studies demonstrate a role for ethylene in the oxidative burst induced by a broad range of stress conditions. Cao, Jiang, and Zhang (2006) reported that paraquat-induced increases in $O_2^{\bullet -}$ and H_2O_2 levels were less pronounced in an *ethylene insensitive 2-1* (*ein2-1*) mutant as compared to those in WT *A. thaliana* plants. Therefore, the extent of lipid peroxidation was significantly lower in the mutant (Cao et al., 2006). Similar responses were described in *ein2-1* mutants exposed to Al (Y.Y. Zhang, He, Zhao, Huang, & Hao, 2014). In addition, blocking of ethylene biosynthesis and/or signalling resulted in a reduced extent of H_2O_2 production in camptothecin-exposed *Lycopersicon esculentum* suspension cells (de Jong, Yakimova, Kapchina, & Woltering, 2002) and

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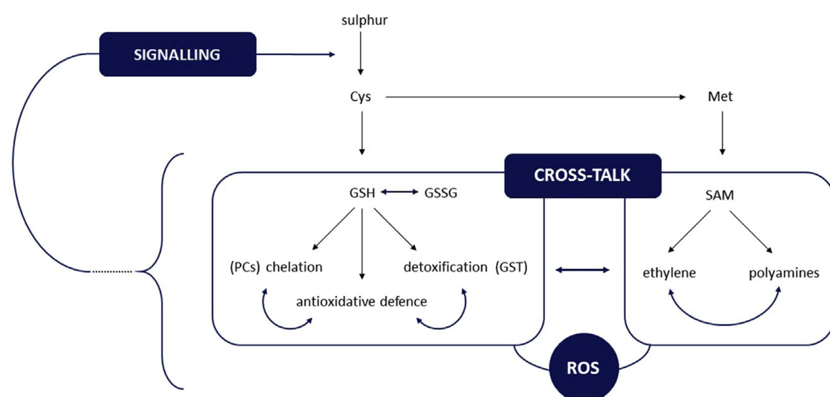

Figure 2 ***Cross talk between detoxification and signal transduction pathways in plants.*** Significant cross talk exists between glutathione-mediated detoxification mechanisms and ethylene- and polyamine-related signal transduction pathways in plants, both under physiological and metal exposure conditions. Reactive oxygen species are put forward as central players in this interaction. Furthermore, several components involved in detoxification and signal transduction pathways are known to affect sulphur uptake and assimilation. *Cys*, cysteine; *GSH*, glutathione; *GSSG*, glutathione disulphide; *GST*, glutathione transferase; *Met*, methionine; *PC*, phytochelatin; *ROS*, reactive oxygen species; *SAM*, S-adenosylmethionine.

Cd-exposed *Phaseolus coccineus* roots (Maksymiec, 2011). Furthermore, inhibition of ethylene biosynthesis limited Cd-induced $O_2^{\bullet -}$ accumulation in root hair tips of *B. napus*, suggesting that ethylene is an upstream regulator of $O_2^{\bullet -}$ generation (Sun & Guo, 2013). The effect of ethylene on $O_2^{\bullet -}$ production is possibly mediated by its interaction with ROS-producing NADPH oxidases, also referred to as respiratory burst oxidase homologues (RBOHs). This is confirmed by the observation that H_2O_2 production induced by the ethylene-releasing compound ethephon in *I. batatas* was limited by treatment with the NADPH inhibitor diphenyleneiodonium (Chen, Huang, Huang, Chow, & Lin, 2013). This relationship between ethylene and NADPH oxidases is also supported under metal exposure conditions, as Hg-induced increases in apoplastic H_2O_2 accumulation and NADPH oxidase activity in apical root segments of *Medicago sativa* were reduced by treatment with an ethylene signalling inhibitor (Montero-Palmero, Martin-Barranco, Escobar, & Hernandez, 2014). Keunen et al. (2015) reported that the Cd-induced *RBOHC* upregulation in several *A. thaliana* ethylene biosynthesis and signal transduction mutants was less pronounced than that in WT plants.

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In addition to its effects on ROS production, ethylene is also linked to several players of the antioxidative defence system. It interacts with both enzymatic antioxidants, such as SOD and CAT, and nonenzymatic antioxidants, such as AsA and α-tocopherol (Keunen, Schellingen, et al., 2016). In the context of this review, the interaction between ethylene and GSH is of particular interest. The cross talk between these two compounds is probably related to the link of ethylene biosynthesis and signalling with sulphate assimilation (Fig. 2), as reviewed by Iqbal et al. (2013) and Wawrzynska, Moniuszko, and Sirko (2015). Indeed, sulphur nutrition has been shown to modulate plant responses to a diverse array of stress factors by increasing ethylene production (Wawrzynska et al., 2015). Nazar, Khan, Iqbal, Masood, and Khan (2014), for example, demonstrated that excess sulphur alleviated the effects of salt stress in *B. juncea*. However, this response was absent when ethylene synthesis was inhibited, suggesting a role for ethylene in the sulphur-induced salt stress alleviation (Nazar et al., 2014). Several genes involved in ethylene signalling were strongly upregulated in sulphate-treated *Vitis vinifera* (Giraud, Ivanova, Gordon, Whelan, & Considine, 2012). In addition to sulphur excess, sulphur limitation was also shown to increase ethylene concentrations and the expression of ethylene-related genes in *N. tabacum* plants (Lewandowska et al., 2010; Moniuszko et al., 2013).

Interestingly, the interplay between ethylene and sulphate assimilation also functions in the other direction, with ethylene levels affecting several proteins of the sulphate assimilation pathway (Fig. 2). It was shown that ethephon significantly induced ATPS activity and sulphur content in *B. juncea* (Iqbal, Khan, Nazar, & da Silva, 2012). Furthermore, treatment of *A. thaliana* with 200 μM ACC was shown to induce APR at the transcriptional and activity level (Koprivova, North, & Kopriva, 2008). As GSH synthesis depends on sulphur availability for the production of its precursor cysteine, ethylene possibly modulates the sulphate assimilation pathway to meet the increasing demand for GSH during stress conditions (Keunen, Schellingen, et al., 2016).

The cross talk between ethylene and GSH is further supported by the observation that application of exogenous GSH in *A. thaliana* increased both gene expression levels and protein abundance of ACS2 and ERF2, involved in ethylene biosynthesis and signal transduction, respectively (Sinha et al., 2015). The ethephon-induced increased ROS production in *I. batatas* was attenuated when plants were treated with exogenous GSH (Chen et al., 2013). Expression levels of ethylene-related genes are often affected in

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transgenic plants with altered GSH levels (Keunen, Schellingen, et al., 2016). Schnaubelt et al. (2015), for example, reported several *ERF* genes to be significantly upregulated in the severely GSH-deficient *rml1-1* *A. thaliana* mutant as compared to WT plants. In contrast, *ERF2* expression was significantly downregulated in the *A. thaliana cad2-1* mutant, which is characterized by a milder GSH deficiency (Han, Mhamdi, Chaouch, & Noctor, 2013). Furthermore, transgenic tobacco plants with an enhanced GSH content displayed a significant upregulation of genes involved in ethylene biosynthesis and signal transduction (Ghanta et al., 2014). Similarly, Datta et al. (2015) reported that transgenic *A. thaliana* plants with elevated GSH levels displayed increased ACS2, ACS6 and ACO1 transcription levels and protein abundance as compared to WT plants, while the opposite response was observed in the GSH-deficient *pad2-1* mutant. The authors demonstrated that the GSH-induced upregulation of *ACS2* and *ACS6* was mediated by a WRKY33-related mechanism, whereas the increased *ACO1* expression was due to an increased stability of its encoding mRNA. Interestingly, application of exogenous GSH also increased the tolerance of WT *A. thaliana* plants to necrotrophic infection and salt stress, while it did not affect the stress tolerance of *ein2-1* mutants deficient in ethylene signalling. These data suggest that GSH induces plant tolerance to different stress factors by an ethylene-dependent mechanism (Datta et al., 2015).

Interestingly, the relationship between ethylene and GSH is also supported during metal exposure (Keunen, Schellingen, et al., 2016). Indeed, ethephon application was shown to increase GSH concentrations in *B. juncea* exposed to Cd (Masood, Iqbal, & Khan, 2012), Ni and Zn (Khan & Khan, 2014). Schellingen et al. (2015) reported that the Cd-induced upregulation of genes involved in GSH biosynthesis was significantly weaker in an *acs2-1/6-1* knockout mutant than in WT *A. thaliana* plants, suggesting that ethylene biosynthesis is essential for the induction of efficient GSH-dependent defence responses during Cd exposure (Schellingen et al., 2015). Similarly, the crucial role of EIN2 in Pb tolerance in *A. thaliana* was also demonstrated to be partially related to its stimulating effect on GSH levels (Cao et al., 2009). The interaction between ethylene and GSH during metal exposure also functions in the other direction, with GSH levels affecting ethylene signalling. Hasan et al. (2016) reported that the Cd-induced upregulation of *ERF1* and *ERF2* was more pronounced in plants supplied with 5 mM GSH, whereas it was weaker in plants treated with the GSH biosynthesis inhibitor buthionine sulphoximine.

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In addition to ethylene, PAs are also subject to cross talk with the antioxidative defence system during stress conditions. Addition of Spd to *Solanum lycopersicum* exposed to chilling stress, for example, strongly enhanced the chilling-induced increase in GSH and AsA levels. Furthermore, it significantly increased the GSH/GSSG and AsA/DHA ratios, possibly as a result of the observed induction of enzymes involved in the AsA-GSH cycle at the transcriptional and/or activity level. While the enzymatic activities of SOD, POD and CAT were decreased by chilling stress, this effect was reversed by adding Spd, with activities even increasing those measured in control plants. These data possibly explain the observed reduction of chilling-induced $O_2^{\bullet -}$ and H_2O_2 production by addition of exogenous Spd (Diao, Song, & Qi, 2015). Similarly, positive effects of PA treatment on the antioxidative defence system were also reported in salt-exposed *O. sativa* (Jain, Vart, Verma, & Malhotra, 2015) and *Cucumis sativus* (Shu, Yuan, Guo, Sun, & Yuan, 2013) and *Glycine max*. subjected to osmotic stress (Radhakrishnan & Lee, 2013).

PAs were also shown to positively affect several components of the antioxidative defence system in metal-exposed plants. Spd treatment was shown to further enhance the Cr-induced increase in GSH levels in *Rhapus sativus*. Furthermore, Spd significantly alleviated Cr-induced lipid peroxidation and H_2O_2 concentrations. In contrast, it counteracted the increase in CAT activity caused by Cr exposure. Overall, exogenous Spd increased plant tolerance to Cr, as indicated by its positive effects on root and shoot length and fresh weight of Cr-exposed plants (Choudhary, Kanwar, Bhardwaj, Yu, & Tran, 2012). Similarly, Rady and Hemida (2015) reported that addition of either Spd or Spm counteracted the negative effects of Cd exposure on several growth parameters of *T. aestivum* plants. This effect was likely related to the fact that PA application attenuated Cd-induced effects on leaf GSH levels and SOD, CAT and APX activity. As a result, Cd-induced lipid peroxidation and H_2O_2 production were reduced in leaves of PA-treated plants. However, these data should be interpreted with caution, as the application of Spd and Spm also significantly reduced shoot Cd concentrations potentially explaining the observed attenuation of metal stress (Rady & Hemida, 2015).

Taken together, available data indicate that significant cross talk exists between cysteine-related detoxification and SAM-related signal transduction pathways under different stress conditions including exposure to metals and possibly also organic contaminants. This interaction should not be surprising, as the sulphur atom of cysteine is used to synthesize methionine

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and its primary metabolite SAM, thereby linking both pathways. However, further research is needed to fully elucidate the mechanisms connecting both pathways (Fig. 2).

6. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The sulphur-containing amino acids cysteine and methionine play crucial roles in plant responses to metals and organic contaminants through their incorporation into the primary metabolites GSH and SAM. GSH is primarily involved in chelation and detoxification mechanisms, whereas SAM mainly contributes to signal transduction reactions mediated by ethylene and PAs. Significant cross talk exists between these detoxification and signal transduction pathways, which is explained by the fact that cysteine is a precursor for both GSH and methionine synthesis. Furthermore, ROS appear to play an important role in mediating the interaction between both pathways. The effects of metal exposure on plant detoxification and signal transduction pathways are relatively well described, whereas information regarding the influence of organic contaminants is rather scarce and mostly limited to herbicides and safeners, indicating a need for further research. Elucidating plant responses to metals and organic compounds can significantly contribute to optimizing phytoremediation strategies for the cleanup of contaminated soils. In this strategy, special attention should be given to sulphur availability in the environment, as it affects sulphate assimilation and downstream detoxification and regulation pathways that are important factors controlling metal uptake and translocation as well as phytotoxic responses. With view to the importance of sulphur nutrition and the internal regulation of sulphur-dependent metabolic pathways for plant health and performance, future research on breeding and practical application of plants should focus on ways to establish stable and adaptive sulphur metabolism in crops. This could be achieved by molecular approaches, fostering plant–microbe interactions or the use of sustainable agrochemical amendments.

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