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Molecular and Cellular Aspects of Contaminant Toxicity in Plants: The Importance of Sulphur and Associated Signalling Pathways Peer-reviewed author version

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1	Running title:	Plant response	es to contaminant	exposure
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3	Molecular and cellular aspects of contaminant toxicity in plants
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12	Abstract
13	Environmental contamination with metals and organic compounds poses a serious threat to human
14	health. Investigating plant responses to these contaminants at the molecular and cellular level is
15	crucial to optimise phytoremediation strategies to clean up contaminated soils. Two key players in
16	plant stress responses are the sulphur-containing amino acids cysteine and methionine. Cysteine is
17	an important constituent of the metal-chelating metallothioneins and is also the precursor for
18	glutathione and subsequent phytochelatin synthesis. During stress conditions, glutathione is
19	involved in (1) metal chelation, (2) xenobiotic detoxification and (3) antioxidative defence. The
20	activated form of methionine, S-adenosylmethionine, is involved in the synthesis of ethylene and

21 polyamines, both playing important roles in signal transduction. This review provides an overview

of sulphur assimilation and its conversion into basic metabolites essential for detoxification and
signal transduction during metal- and organic contaminant-induced stress conditions in plants.
Furthermore, the cross-talk between these pathways and their relation to the contaminant-induced
oxidative challenge are discussed.

26

27 1 General introduction

Since the industrial revolution, but especially over the last decades, abiotic contaminants – mainly 28 from agricultural and industrial origin - have emerged into the environment and are recognised to 29 pose a serious threat to the environment and human health (Ibrahim, Hayyan, AlSaadi, Hayyan, & 30 Ibrahim, 2016; Noguera-Oviedo & Aga, 2016). These contaminants can be categorised in different 31 32 groups based on their physicochemical properties, but their persistence (e.g. metals) and degradability (e.g. organics) are determining factors for their residence in the environment. As a 33 result, different strategies are needed to remediate the environment. Microbe-assisted 34 phytoremediation is a promising technology to be implemented in the clean-up of soils polluted 35 with metals and organics (Chirakkara, Cameselle, & Reddy, 2016; Gerhardt, Huang, Glick, & 36 Greenberg, 2009; Ma, Oliveira, Freitas, & Zhang, 2016; Vangronsveld et al., 2009). However, to 37 exploit this technology, it is important to unravel the underlying molecular and cellular responses 38 of plants in order to minimise phytotoxic responses. 39

40 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, 41 Antosiewisz, Schwitzguébel, & Schröder, 2009). More recently, multiple studies have focussed on 42 43 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 44 and (3) sequestration, all of which should be highly coordinated to prevent cellular damage. In 45 addition to the detoxification of excess contaminants, also other factors such as environmental 46 conditions (e.g. temperature, humidity) determine the stress intensity perceived by the plant 47 (Reichenauer & Germida, 2008). Pivotal modulators of plant metabolism upon multiple 48

environmental challenges are reactive oxygen species (ROS), involved in oxidative damage but
also 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).

53 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 54 55 the metal chelators metallothioneins (MTs), glutathione (GSH) and phytochelatins (PCs) (Anjum, Gill, & Gill, 2014). Additionally, GSH is often consumed in conjugation reactions of organic 56 compounds prior to sequestration into the vacuole (Dixon, Skipsey, & Edwards, 2010). Besides its 57 role in detoxification, GSH is a major cellular antioxidant in plants (Noctor et al., 2012). As such, 58 59 it is clear that detoxification and regulation are closely interconnected in plant stress acclimation. 60 Methionine, the other sulphur-containing amino acid, forms the basis of S-adenosylmethionine 61 (SAM), a central component in plant metabolism. It is also the initial biosynthetic compound in the 62 production of the stress hormone ethylene (Sauter, Moffatt, Saechao, Hell, & Wirtz, 2013). Besides 63 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, 2016b). 64 From this, it becomes clear that upon exposure to excess organics or metals, sulphur is an essential 65 66 element in (1) detoxification pathways and (2) the regulation of plant stress responses. Therefore, the current review focusses on sulphur uptake and assimilation and its conversion into basic 67 metabolites essential for detoxification and regulation mechanisms under organic and metal 68 69 exposure in plants. Via these sulphur-containing metabolites, detoxification and regulation are strongly interconnected and are discussed in view of the oxidative challenge and signal 70 71 transduction under stress conditions in plants.

72 2 Sulphur uptake and assimilation

Sulphur is an essential macronutrient for all organisms, serving different metabolic functions. It is 73 incorporated into the amino acids cysteine and methionine, controlling the structure and biological 74 activity of many proteins (Davidian & Kopriva, 2010; Gotor et al., 2015). Furthermore, it is an 75 important component of several coenzymes and prosthetic groups and is involved in plant 76 77 responses to both biotic and abiotic stress factors (Davidian & Kopriva, 2010; Gigolashvili & Kopriva, 2014; Romero et al., 2014). In soil, sulphur is mainly present as inorganic sulphate (SO₄²⁻ 78). In order to enter metabolic pathways, sulphate is assimilated in plants through a pathway 79 80 consisting of (1) sulphate uptake, (2) sulphate activation, (3) sulphate reduction and (4) synthesis of cysteine (Fig. 1) (Droux, 2004; Ravilious & Jez, 2012). 81

82 [Insert Figure 1 here]

Sulphate is taken up by plants via the action of proton/sulphate cotransport systems (Gigolashvili 83 & Kopriva, 2014; Takahashi, Kopriva, Giordano, Saito, & Hell, 2011). The plant family of sulphate 84 85 transporters (SULTRs), comprised of 12 to 16 genes depending on the plant species, is subdivided into four functional groups based on sequence, biochemical characteristics and physiological 86 function (Takahashi et al., 2011). The SULTR family is best characterised in the model organism 87 Arabidopsis thaliana (Gigolashvili & Kopriva, 2014). The most important transporters involved in 88 sulphate uptake in roots are the high-affinity SULTR1;1 and SULTR1;2 proteins (group 1). 89 90 However, as the expression of the SULTR1;1 gene in roots is much lower as compared to that of 91 SULTR1,2, the latter is considered the most important transporter involved in plant sulphate uptake 92 during normal sulphate supply (Gigolashvili & Kopriva, 2014; Rouached et al., 2008). During 93 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-94 95 Nakashita et al., 2005). Another known sulphate transporter is SULTR1;3, which is localised in phloem companion cells and involved in sulphate transport from source to sink (Yoshimoto, Inoue, 96 Saito, Yamaya, & Takahashi, 2003). Furthermore, the low-affinity group 2 SULTRs facilitate long 97 distance sulphate transport (Gigolashvili & Kopriva, 2014; Takahashi et al., 2000). The activity of 98 SULTR2;1 is modulated by SULTR3;5, which does not transport sulphate itself (Kataoka, Hayashi, 99 100 Yamaya, & Takahashi, 2004a). In contrast to SULTR3;5, other group 3 SULTRs function in sulphate import into plastids (Cao et al., 2013; Gigolashvili & Kopriva, 2014), as the enzymes 101 necessary for sulphate reduction are exclusively present in these organelles (Davidian & Kopriva, 102 2010). In addition, transporters belonging to group 4 are localised in the tonoplast, releasing 103 sulphate from vacuoles (Kataoka et al., 2004b; Takahashi et al., 2011). 104

105 Once taken up in plant cells, sulphate is activated in an adenylation reaction catalysed by ATP 106 sulphurylase (ATPS). The resulting adenosine 5'-phosphosulphate (APS) is an important 107 branching point in the sulphate assimilation pathway (Fig. 1). It can be phosphorylated by the 108 action of APS kinase (APK), leading to the formation of 3'-phosphoadenosine 5'-phosphosulphate 109 (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 110 APS to sulphite (SO_3^{2-}) , which is further reduced to sulphide (S^{2-}) by the ferredoxin-dependent 111 sulphite reductase (SiR). Sulphide is subsequently used for the synthesis of cysteine (Droux, 2004; 112 113 Takahashi et al., 2011). While ATPS is also present in the cytosol, the reductive steps of the 114 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 117 118 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). 119 In the first and rate-limiting step of cysteine biosynthesis, O-acetylserine (OAS) is synthesised 120 121 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 122 sulphide in a β-replacement reaction catalysed by OAS (thiol) lyase (OASTL). Both SAT and 123 OASTL are present in the cytosol, plastids and mitochondria (Anjum et al., 2014). These enzymes 124 were demonstrated to associate, forming the hetero-oligomeric cysteine synthase complex. This 125 126 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 127 to SAT, suggesting that this enzyme only functions as a chaperone-like subunit in the cysteine 128 129 synthase complex (Wirtz et al., 2010; Wirtz & Hell, 2006). However, as cellular OASTL activity is 100 to 300 times higher as compared to SAT activity, free OASTL enzymes are present in cells 130 and catalyse the formation of cysteine from sulphide and OAS released by the complex (Droux, 131 2004; Saito, 2000). It must be noted that several metabolites regulate the stability of the cysteine 132 synthase complex. Under sulphur deprivation conditions, for example, increased OAS levels 133 stimulate dissociation of the complex, thereby inhibiting SAT activity and further OAS 134 accumulation. In contrast, the presence of sulphide counteracts the dissociation of SAT and 135 OASTL, resulting in the maintenance of SAT activity as long as sulphide availability is not a 136 137 limiting factor (Droux, Ruffet, Douce, & Job, 1998; Takahashi et al., 2011; Wirtz & Hell, 2006).

138 2.1 Response to metal stress

In addition to its regulation by internal stimuli, the sulphate assimilation pathway is affected by 139 140 environmental factors including metal exposure (Ernst, Krauss, Verkleij, & Wesenberg, 2008; Na 141 & Salt, 2011). Indeed, several metals were shown to influence the expression of genes encoding both high- and low-affinity sulphate transporters, thereby affecting sulphate uptake and 142 143 assimilation. Nocito et al. (2006), for example, demonstrated that exposure to different 144 concentrations of cadmium (Cd), copper (Cu) and zinc (Zn) increased the expression of ST1;1, a high-affinity sulphate transporter in Zea mays. As a result, sulphate uptake capacity in roots was 145 significantly enhanced under all metal exposure conditions (Nocito et al., 2006). Furthermore, Zn 146 147 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) 148 149 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 150 accumulation and translocation. As a consequence, cysteine, GSH and PC levels were higher in 151 both roots and shoots (Dixit et al., 2016). However, exposure to chromium (Cr) clearly decreased 152 the expression levels of ST1;1 and significantly suppressed sulphate uptake in Z. mays (Schiavon 153 154 et al., 2007). Furthermore, Cr was also shown to reduce sulphate uptake and negatively affect the transcription of several sulphate transporters including SULTR1;3, SULTR2;1, SULTR3;2, 155 SULTR3;5 and SULTR4;1 in roots of Brassica juncea after 24 hours of exposure. In contrast, 156 expression levels of SULTR4:2 were significantly increased at this time point (Schiavon et al., 157 2012). 158

In addition to their effect on sulphate uptake, metals also influence transcript levels, proteinabundance and activity of enzymes catalysing different reactions in the sulphate assimilation

pathway. For example, an increased activity of the sulphate-activating enzyme ATPS was reported 161 162 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 163 and shoot ATPS transcript levels in a concentration-dependent manner. Moreover, the expression 164 165 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, also Cu was shown 166 to affect sulphate assimilation, as demonstrated by a strongly increased ATPS expression in roots 167 of both A. thaliana and Arabidopsis halleri (Weber, Trampczynska, & Clemens, 2006). 168 Furthermore, Cu-induced increases in the protein abundance of APR, SAT and OASTL in O. sativa 169 170 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 171 al., 2013; Liang et al., 2014; Nocito et al., 2006), Cr (Schiavon et al., 2012; Schiavon, Pilon-Smits, 172 Wirtz, Hell, & Malagoli, 2008), Cu (Gajewska, Glowacki, Mazur, & Sklodowska, 2013), nickel 173 (Ni) (Gajewska et al., 2013), lead (Pb) (Mandavian, Ghaderian, & Schat, 2016) and Zn (Stuiver et 174 al., 2014) were shown to alter cysteine levels in a wide range of plant species. 175

However, it is important to note that plant responses to metal exposure depend on sulphate supply, 176 177 as metal-induced effects observed during sulphate starvation conditions often differ from those 178 observed during normal sulphate availability. Indeed, results of Bashir et al. (2015) indicate that 179 under conditions of sulphate starvation, B. juncea displayed higher Cd-induced oxidative stress 180 levels and an increased Cd sensitivity. Furthermore, sulphur addition was reported to increase Cd 181 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 182 the Cd-induced inhibition of root and shoot growth and significantly reduced malondialdehyde 183

(MDA) and superoxide (O_2^{\bullet}) levels in both organs (Liang et al., 2016). Furthermore, decreases in *Corchorus olitorius* dry weight induced by aluminium (Al), Cd, Cu and Pb were alleviated by the addition of either K₂SO₄ 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 189 190 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 191 OAS, cysteine and GSH levels in A. thaliana, thereby increasing its resistance to Ni-induced 192 growth inhibition (Freeman et al., 2004). Furthermore, overexpression of different OASTL isoforms 193 194 increased Cd tolerance of both A. thaliana and Nicotiana tabacum (Dominguez-Solis, Gutierrez-195 Alcala, Romero, & Gotor, 2001; Dominguez-Solis et al., 2004; Harada, Choi, Tsuchisaka, Obata, 196 & Sano, 2001; Kawashima et al., 2004; Ning, Zhang, Yao, & Yu, 2010). Interestingly, transgenic 197 Brassica napus plants overexpressing miR395 also showed diminished Cd-induced oxidative stress 198 levels and were more tolerant to Cd. This miRNA is induced by sulphate starvation and controls 199 the expression levels of SULTR2;1 and three ATPS isoforms. However, expression of miRNA395 is also increased under Cd exposure conditions, highlighting the similarity between plant responses 200 201 to both stress factors (Gielen, Remans, Vangronsveld, & Cuypers, 2016; Zhang, Song, Shu, Zhang, 202 & Yang, 2013).

Another finding indicating the importance of sulphate assimilation in plant responses to metal stress is the fact that transcript levels and activity of enzymes involved in sulphate uptake often differ between metal hyperaccumulators and their non-accumulator counterparts. Freeman et al. (2004), for example, reported strong positive correlations between OAS, cysteine and GSH levels

and shoot Ni concentrations in various *Thlaspi* hyperaccumulators and non-accumulators. Indeed, 207 208 the metal-accumulating species *Thlaspi goesingense* displayed increased SAT activity and OAS, 209 cysteine and GSH levels in comparison to the related non-accumulator A. thaliana under control conditions. When exposed to Ni, this resulted in an increased resistance of the former species 210 211 against Ni-induced oxidative stress, as indicated by considerably lower concentrations of thiobarbituric acid-reactive substances (TBARS) (Freeman et al., 2004). Furthermore, metal-212 tolerant genotypes of the same species often exhibit increased sulphate assimilation capacities as 213 214 compared to their metal-sensitive counterparts. For example, in *Lens culinaris*, an As-tolerant cultivar, showed strongly increased SAT and OASTL transcript levels and activities and 215 significantly enhanced cysteine and GSH levels as compared to an As-sensitive cultivar exposed 216 to As. Interestingly, As differently affected transcript levels of group 1 sulphate transporters 217 between both cultivars, with SULTR1;1 and SULTR1;2 upregulation in the tolerant and 218 219 downregulation in the sensitive cultivar after 24 hours of exposure (Talukdar & Talukdar, 2014). Similarly, Song et al. (2013) reported more pronounced Cu-induced increases in SAT and OASTL 220 protein abundance in a Cu-tolerant as compared to a Cu-sensitive O. sativa variety. Furthermore, 221 222 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 223 obvious that sulphate uptake and assimilation play a prominent role in plant responses to metal 224 stress. This can be explained by the crucial role of cysteine-containing compounds in metal 225 226 chelation and detoxification, as discussed in section 3.

227

228 2.2 Response to organic contaminants

While our knowledge on the effects of organic environmental contaminants on sulphur assimilation 229 230 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 231 1985, the lab of Lamoureux described that pretreatment of Z. mays with low levels of the 232 233 chloroacetamide herbicide 2-chloro-N,N-di-2-propenylacetamide protected them from the 234 herbicide when it was applied later at higher concentrations by increasing GSH synthesis (Ezra, Rusness, Lamoureux, & Stephenson, 1985). Similarly, dichloroacetamide herbicide antidotes were 235 shown to enhance sulphate metabolism in Z. mays roots by Adams et al. (1983). Diclormid and 236 benoxacor both induced ATPS, whereas flurazole acted on OASTL (Hirase & Molin, 2003). Such 237 238 an effect was also observed a few years later for Sorghum bicolor by Gronwald and co-workers 239 (1987). In 1990, evidence for a direct interaction of N,N-diallyl-2,2-dichloroacetamide and 4dichloroacetyl-3,4-dihydro-3-methyl-2H-1,4-benzo-oxazin (CGA 154 281) with sulphate 240 241 assimilation and GSH contents was presented in Z. mays (Farago & Brunold, 1990). Similar to the safener CGA 154 281, cysteine formation and GSH synthesis were also induced by the safener 1-242 dichloroacetyl-hexahydro-3,3,8-trimethyl-pyrrolo-[1,2-]-pyrimidine-6-(2H)-one-(dicyclonone) 243 244 (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 245 unravelled yet. Just recently, the effect of three herbicide safeners (mefenpyr-diethyl, 246 fenchlorazole-ethyl and dichlormid) on the content of sulphur-containing metabolites in Fe-247 248 deficient barley was investigated (Bartucca et al., 2016). All three safeners were effectively 249 inducing ATPS activity, but the effect on OASTL activity was dependent on concentration as well 250 as exposure time. An initial reduction of OASTL activity was followed by a strong induction. The authors speculate 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).

258

259 **3** Thiols play an important role in detoxification

260 3.1 Metallothioneins

261 Metallothioneins 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 262 groups based on the number and arrangement of their cysteine residues (Cobbett & Goldsbrough, 263 264 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, 265 Imam, & Blindauer, 2013). The cysteine-rich α - and β -domains are involved in metal binding. The 266 267 α-domain is located at the C-terminus of the protein and contains six cysteine residues arranged 268 according to the consensus sequence CxCxxxCxCxCxCxC, where x represents any other amino acid than cysteine. The β-domain is present at the N-terminus and has a more variable amino acid 269 sequence. It generally contains six cysteine residues in MT1 proteins, eight in MT2 proteins and 270 four in MT3 proteins. Type 4 MTs – also referred to as E_c proteins – can be distinguished from 271

other MT classes based on the presence of three cysteine-rich domains separated by two linker regions. Furthermore, they are characterised 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). While 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 279 are more strongly expressed in roots as compared to shoots, whereas the opposite holds true for 280 type 2 MTs (Guo, Bundithya, & Goldsbrough, 2003). Although type 3 MTs are also present in 281 282 leaves, they are mainly expressed in ripening, fleshy fruits (Clendennen & May, 1997; Moyle, 283 Fairbairn, Ripi, Crowe, & Botella, 2005). In contrast, MTs of type 4 are exclusively localised in 284 developing seeds, implying a role in metal storage and accumulation there (Guo et al., 2003). As 285 proposed by Ren et al. (2012), these MTs could provide a means to store Zn required for seed 286 germination. Furthermore, different MT genes are strongly upregulated in ageing leaves, 287 suggesting their involvement in leaf senescence. During this process, MTs possibly serve a dual function. On one hand, they could protect cells against metal toxicity arising from the breakdown 288 289 of different metal-containing cellular components. On the other hand, they could also be involved 290 in translocating the released metals to non-senescing plant tissues (Guo et al., 2003; Leszczyszyn 291 et al., 2013).

Although many questions remain with regard to the different functions of plant MTs, their role in metal chelation is well established. Metallothioneins have the ability to bind both mono- and divalent metal ions in typical metal-thiolate clusters, characterised by high thermodynamic and low

kinetic stability. As a consequence, metals are tightly bound by MTs, but part of the metal ions can 295 296 be readily relocated to other proteins (Hassinen, Tervahauta, Schat, & Karenlampi, 2011). Both essential and non-essential metals are chelated by MTs, indicating their involvement in nutrient 297 298 homeostasis as well as metal detoxification. The metals Zn, Cu and Cd are bound to MTs with the 299 highest affinity (Blindauer & Leszczyszyn, 2010). The importance of MTs in detoxifying excess 300 metals is highlighted by their induction under metal exposure conditions, as discussed in the following section (3.1.1). However, many other biotic and abiotic stress factors including pathogen 301 302 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 303 304 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 305 the promoter regions of genes encoding these proteins. Indeed, metal-, antioxidant-, wounding-and 306 307 stress-responsive elements were identified in the promoters of type 1, 2 and 3 MTs. Furthermore, elements responsive to different phytohormones including ethylene, methyl jasmonate, gibberellic 308 309 acid and salicylic acid were also found in MT promoters, suggesting hormonal regulation of MT 310 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), reporting increased stress tolerance and decreased H_2O_2 levels in transgenic *N. tabacum* plants overexpressing the *Gossypium hirsutum MT3a* gene as compared to the wildtype (WT) exposed to cold, salt and drought stress. Moreover, 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 318 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) 319 increased the expression of several MT genes in many plant species including Quercus suber (Mir 320 321 et al., 2004), G. hirsutum (Xue et al., 2009), Ipomoea batatas (Kim, Jeong, Ahn, Lee, & Kwak, 322 2014) and O. sativa (Liu et al., 2015a). The antioxidative function of MTs can be explained by the 323 fact that the cysteine residues responsible for metal scavenging also have the capability to reduce ROS. During metal exposure conditions, MTs could also exert an indirect antioxidative function 324 by chelating redox-active metals such as Cu, thereby preventing ROS formation as a consequence 325 326 of Fenton and Haber-Weiss reactions (Leszczyszyn et al., 2013).

327

328 3.1.1 <u>Response to metal stress</u>

As mentioned before, metal exposure induces MT expression in numerous plant species. Exposure 329 to Cu, for example, induced the expression of MT1a, MT2a, MT2b and MT3 in different organs of 330 331 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 332 significantly induced the expression of MT2a in A. thaliana roots and leaves (Jozefczak et al., 333 334 2014). In another study, 24 hours exposure to Cd, Cu or Zn was shown to induce the expression of MT1 in Cajanus cajan (Sekhar, Priyanka, Reddy, & Rao, 2011). Furthermore, these metals also 335 336 increased MT3 transcription levels in leaves of Porteresia coarctata (Usha, Keeran, Harikrishnan, 337 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, 338 strongly induced MT2a and MT2b expression in shoots of Hirschfeldia incana (Auguy et al., 2016). 339 Similarly, MT1 and MT2 expression levels were elevated in As-exposed O. sativa (Nath et al., 340

2014). Furthermore, also 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 346 347 in MT expression between metal-sensitive plants and their tolerant counterparts. Indeed, MT3 transcript levels were approximately 2.5-fold higher in the metal hyperaccumulator Thlaspi 348 caerulescens as compared to the non-accumulator A. thaliana grown under control conditions. 349 Furthermore, the cysteine positions in the MT amino acid sequence of both species were different, 350 351 predicting a smaller metal binding cavity in A. thaliana as compared to T. caerulescens (Roosens, Bernard, Leplae, & Verbruggen, 2004). Interestingly, MT2a, MT2b and MT3 expression levels 352 353 were also 1.5- to 4-fold higher in the metal hyperaccumulator A. halleri as compared to those in A. 354 thaliana (Chiang, Lo, & Yeh, 2006). In addition, MT2b expression was considerably higher in two 355 independently evolved Cu-tolerant Silene paradoxa populations as compared to a Cu-sensitive 356 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). 357 358 Furthermore, Hassinen et al. (2009) reported that MT2 and MT3 were more highly expressed in 359 metallicolous T. caerulescens accessions as compared to a non-metallicolous accession. However, 360 Zn accumulation and MT transcript levels did not co-segregate, implying that MTs are not the 361 major determinants of Zn accumulation in these plants.

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, 364 365 Cu and Zn exposure on root growth of N. tabacum seedlings. Furthermore, it significantly increased metal accumulation in these plants. The positive function of this MT during metal stress could be 366 related to its metal-chelating function, but could also be associated with its antioxidant properties. 367 368 This is confirmed by significantly reduced H_2O_2 and lipid peroxidation levels in the CeMT-369 overexpressing plants as compared to their WT counterparts after 24 hours of metal exposure (Kim, Jung, Kim, & Bae, 2013). Similarly, overexpression of MT2 from S. alfredii resulted in increased 370 371 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 372 373 WT. Furthermore, when exposed to Cd and Zn, the MT2 overexpressing plants also displayed 374 increased SOD, CAT and POD activities in comparison to WT plants (Zhang et al., 2014a). In addition, overexpression of MTs from yeast and mammals can also increase metal tolerance and/or 375 376 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. 377 378 Although MTs are important players in plant metal stress responses, it should be noted that their 379 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 380 381 upregulated by Cu and Zn exposure and downregulated by Cd exposure in Hordeum vulgare. 382 Similarly, transcript levels of MT2c and MT3 increased in response to Cu, while they decreased in 383 leaves of Cd-exposed plants. In contrast, expression levels of these MTs remained unchanged in 384 response to Zn. Furthermore - as mentioned above - expression levels of different MTs were strongly tissue-dependent, with MT1b and MT4 transcripts exclusively detected in roots and grains 385 respectively (Schiller et al., 2014). Taken together, these data point towards distinct physiological 386

functions for different MT isoforms. A first step towards unravelling these functions involves the analysis of MTs at the protein level. This remains a challenge, however, as plant MTs are very prone to proteolysis, hampering the application of proteomics approaches (Blindauer & Leszczyszyn, 2010).

391

392 3.2 Glutathione

Glutathione is the most abundant non-protein thiol in almost all aerobic species, occurring at 393 394 intracellular concentrations of 0.5 to 10 mM. It is a tripeptide consisting of γ -glutamate, cysteine 395 and glycine and is synthesised 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 catalysed 396 by γ -EC synthetase (γ -ECS; GSH1), also known as glutamate-cysteine ligase (GCL), and 397 constitutes the rate-limiting step in GSH biosynthesis. Subsequently, GSH is formed by the 398 addition of glycine to γ -EC in a reaction catalysed by glutathione synthetase (GSH-S; GSH2) 399 400 (Noctor et al., 2012). While GSH1 is exclusively localised in chloroplasts, GSH2 is also present in the cytosol, with cytosolic GSH2 activity strongly exceeding that in chloroplasts (Wachter, Wolf, 401 Steininger, Bogs, & Rausch, 2005). Transport of γ -EC into the cytosol, necessary for cytosolic 402 403 GSH production, is mediated by chloroquine-resistance transporter-like transporters (CLTs) in the plastid envelope (Maughan et al., 2010). Once synthesised, GSH is distributed to different 404 405 organelles by the action of plastidal CLTs and transporters in mitochondrial and nuclear membranes (Zechmann, 2014). Mitochondria, chloroplasts and the cytosol have the highest GSH 406 abundance in plant cells (Noctor et al., 2012). In addition to its intracellular transport, GSH is also 407 transported between cells and is one of the major reduced sulphur forms translocated in the phloem 408 (Noctor et al., 2012). 409

An important determinant of GSH biosynthesis is GSH1 activity, which is regulated at three levels. 410 411 First, GSH1 activity is subject to feedback inhibition by GSH itself. Under stress conditions, GSH 412 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-413 414 binding protein with the 5'-untranslated region of its encoding gene (Noctor, Gomez, Vanacker, & Foyer, 2002). Furthermore, GSH1 is post-transcriptionally modulated by the cellular redox state. 415 Under oxidising conditions, it functions as a homodimeric enzyme with two intermolecular 416 disulphide bonds. Reducing conditions however, disrupt one of these disulphide bonds, thereby 417 altering the dimer interface and shifting the enzyme to its less active monomeric form (Hothorn et 418 al., 2006). Whether this mechanism is responsible for GSH1 feedback inhibition by GSH is 419 420 currently unknown (Jozefczak, Remans, Vangronsveld, & Cuypers, 2012). It is interesting to note that GSH biosynthesis is tightly coupled to sulphate assimilation, as stress-induced GSH 421 422 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; 423 424 Smith, Kendall, Keys, Turner, & Lea, 1985).

425 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 426 427 is underlined by the observation that plants lacking GSH due to a knockout mutation in either GSH1 428 or GSH2 display a lethal phenotype (Cairns, Pasternak, Wachter, Cobbett, & Meyer, 2006; Lim, Meyer, & Cobbett, 2011). In contrast, plants with lowered GSH levels are viable but show an 429 430 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 431 (Cheng, Seeley, & Sung, 1995; Vernoux et al., 2000). Other GSH-deficient mutants such as 432

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, emphasising the
crucial role of GSH during stress conditions (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).

441

442 3.2.1 <u>Glutathione in chelation: Phytochelatins</u>

443 An important role of GSH during stress relies on its function as a precursor of PCs. These molecules 444 are important metal scavengers in plant cells and are characterised by a typical $(\gamma$ -Glu-Cys)_n-Gly 445 structure, with n ranging from 2 to 11 (Zagorchev, Seal, Kranner, & Odjakova, 2013). 446 Phytochelatins are synthesised by PC synthase (PCS) in a two-step reaction. In the first step, the 447 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 448 449 or an oligomeric PC peptide – in a transpeptidation reaction. Even though its encoding gene is constitutively expressed, PCS activity is dependent on the presence of metals. While Cd is the most 450 451 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 452 the presence of four highly conserved cysteine residues in 453 related to is its N-terminal domain, which also contains the active site. The C-terminal domain, in contrast, is rich 454

in cysteine residues that possibly bind metal ions in order 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 upon
Cd exposure (Brunetti et al., 2015).

461 As mentioned above, 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 462 mutants cad1-3 and cad1-6 – carrying a mutation in the PCS1 gene – display an enhanced 463 sensitivity towards several metals such as Cd, Pb and Zn (Fischer, Kuhnlenz, Thieme, Schmidt, & 464 465 Clemens, 2014; Tennstedt, Peisker, Bottcher, Trampczynska, & Clemens, 2009). Furthermore, 466 differences in the extent of PC synthesis between metal-sensitive and -tolerant varieties are often observed. Indeed, a Cd-tolerant Triticum aestivum cultivar displayed stronger Cd-induced 467 468 increases in GSH levels and PCS expression in comparison to a Cd-sensitive cultivar (Kumari, 469 Parmar, & Sharma, 2015). Similarly, *PCS1* expression and total PC levels were significantly 470 increased by As exposure in an As-tolerant O. sativa cultivar, while they remained unaffected in its As-sensitive counterpart. In addition, As-induced increases in GSH, cysteine and methionine 471 472 levels were also more pronounced in the tolerant as compared to the sensitive cultivar (Begum et 473 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 as compared to the metal hyperaccumulator *T*.

caerulescens (Martínez et al., 2006). The use of transgenic plants with increased PC production 478 479 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 480 can cause a strong decrease of cellular GSH levels. Since GSH also serves important antioxidative 481 482 functions (see section 3.2.3), GSH depletion possibly results in oxidative stress and cellular damage. This was shown by Jozefczak and co-workers (2014), who reported a rapid increase in PC 483 levels in roots and leaves of A. thaliana plants exposed to 5 and 10 µM Cd. In roots, this response 484 coincided with a fast (2 hours) reduction of GSH levels. Despite the activation of alternative 485 antioxidative defence systems after 24 hours, decreased biomass and increased lipid peroxidation 486 were observed in Cd-exposed plants (Jozefczak et al., 2014). These results indicate that plant metal 487 tolerance is not only related to the production of PCs, but also to the ability to prevent the resulting 488 GSH depletion. Therefore, the use of transgenic plants overexpressing both PCS and GSH 489 biosynthesis genes could be more efficient to remediate metal-contaminated areas (Seth et al., 490 2012). 491

492

493

3.2.2 <u>Glutathione in detoxification: glutathione transferases</u>

Another important function of GSH is related to its involvement in detoxification reactions mediated by GSTs (Fig. 1). These enzymes catalyse the conjugation of xenobiotics to GSH by addition or substitution reactions in order 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 characterised by the presence of two carbon atoms coupled by a 501 502 double bond, adjacent to an electron withdrawing group (Frova, 2003). Given the reactivity of these 503 molecules, conjugation to GSH occurs spontaneously but is speeded up considerably by GST activity (Coleman et al., 1997). In addition to their role in GSH conjugation reactions, GSTs also 504 505 serve a broad range of other catalytic as well as non-catalytic functions (Frova, 2003). While the 506 molecular structure and catalytic mechanisms of GSTs have been studied by several research groups, their natural functions remains largely obscure. An overview of natural GST functions 507 elucidated so far is provided in Table 1. It is interesting to note that the first functions ascribed to 508 GSTs were all related to conjugation (e.g. of xenobiotics), whereas more sophisticated functions 509 510 were only discovered more recently.

511 [Insert Table 1 here]

512 Even though most GSTs are soluble proteins present in the cytosol, they have also been detected 513 in several organelles including chloroplasts, mitochondria, peroxisomes, the vacuole and the 514 nucleus. Furthermore, microsomal GSTs with herbicide detoxifying activities have been characterized in Pachyrhizus erosus (Belford, Dorfler, Stampfl, & Schroder, 2004) and in lower 515 516 plants (Pflugmacher, Schroder, & Sandermann, 2000). Microsomal GSTs belonging to the 'Membrane Associated Proteins involved in Eicosanoid and Glutathione metabolism' (MAPEG) 517 518 family have been characterised as well (Oztetik, 2008). It is important to note that cytosolic GSTs are very abundant, constituting up to 2% of all soluble proteins in plants (Scalla & Roulet, 2002). 519

To date, plant GSTs are subdivided into several classes according to a classification system proposed by Droog (1997) and subsequently refined by Edwards and co-workers (2000). Using this system, GSTs are classified based on gene organisation (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, 524 525 they are also predominantly active in xenobiotic detoxification (Frova 2006, Edwards et al. 2011). Indeed, phi class GSTs have been co-crystallised with a range of herbicide and other xenobiotic 526 conjugates (Prade, Huber, & Bieseler, 1998). In contrast, the zeta (GSTZ) and theta (GSTT) 527 528 families are much smaller and are related to mammalian GSTs. While GSTs belonging to these classes display GSH-conjugating activities, other classes including the lambda GSTs (GSTL) and 529 dehydroascorbate reductases (DHARs) have no known ability to conjugate or detoxify synthetic 530 compounds. Instead, they seem to have a redox-related role (Dixon, Davis, & Edwards, 2002). 531

Structurally, most cytosolic GSTs occur as dimers consisting of two subunits (approximately 25 532 kDa each), each encoded by one gene (Oztetik, 2008). Although homodimers and heterodimers can 533 534 be formed, dimerisation has only been observed between GSTs from the same class (Armstrong, 535 1997). An exception are the DHARs and lambda GSTs, which function as monomers (Dixon et al., 536 2010). Each GST subunit is characterised by a common overall structure with a well-defined N-537 terminal GSH binding domain (G-site) and a less specific C-terminal domain binding the 538 hydrophobic co-substrate (H-site). Most GST classes including the phi, tau, zeta and theta class 539 contain a serine in the active site, responsible for the formation and stabilisation of the reactive thiolate anion from GSH, which is the target for the nucleophilic attack of an electrophilic substrate 540 541 (Dixon et al., 2010). In DHARs and lambda GSTs however, this serine is replaced by a cysteine 542 that forms a mixed disulphide with GSH. The catalytic cysteine performs a nucleophilic attack on 543 GSH-conjugated substrates. Under these conditions, the catalytic cysteine of the GST becomes 544 glutathionylated, thereby deconjugating the substrate that is subsequently released. Regeneration of the resulting glutathionylated GST forms requires a GSH molecule, forming GSSG as another 545 end product. While the reduced GSTs can be used in another catalytic cycle, GSSG is reduced back 546

to GSH by glutathione reductase (GR) at the expense of NADPH (Lallement, Brouwer, Keech,
Hecker, & Rouhier, 2014).

In general, phi and tau GST-catalysed substitution reactions change the parent compound to an 549 550 extent that the toxicity of the xenobiotic is significantly lowered (Schroder & Collins, 2002). Along 551 with the resulting increase in polarity, GSH addition is a very effective means of detoxification, 552 especially when the electrophilic centre of the target molecule is a leaving group. In some 553 situations, however, the formation of 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 554 accumulating in the cytosol inhibit the stress-reducing activity of GR (Schroder & Pflugmacher, 555 1996). Furthermore, when detoxification reactions withdraw GSH from the cellular pool, GSH 556 557 depletion due to exaggerated utilisation in conjugation reactions or inhibition of GSH supply could 558 become a problem, particularly when the xenobiotic conjugates still remain reactive and/or are not 559 efficiently stabilised (Brazier-Hicks et al., 2008). However, GS-conjugate formation has generally 560 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 ATP-binding cassette 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).

567 Interestingly, GSTs are induced by a broad range of biotic and abiotic stress conditions, including 568 exposure to organic contaminants. The relevance of this induction is mostly related to direct 569 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).

572

573 3.2.2.1 Response to stress conditions

574 As mentioned above, GSTs are important players in plant responses to organic compounds. In this 575 context, especially herbicides are known to affect plant GST levels and activities. For example, 576 glyphosate significantly increased GST activities in *Pisum sativum* roots and leaves (Miteva, 577 Ivanov, & Alexieva, 2010). Similarly, exposure to the chloroacetamide herbicide metazachlor 578 induced GST activity in B. napus leaves (Vercampt, Koleva, Vassilev, Vangronsveld, & Cuypers, 2016). A role for GSTs in herbicide detoxification is further supported by the enhanced tolerance 579 of N. tabacum plants overexpressing a Citrus cinensis tau class GST to the diphenyl ether herbicide 580 fluorodifen. Interestingly, salt and drought tolerance were also increased in the overexpressor 581 582 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, 583 Madesis, Tsaftaris, & Lo Piero, 2015). Even though GSTs play an important role in detoxifying 584 herbicides, their activities are not always induced in herbicide-exposed plants. Indeed, the 585 586 photosynthetic herbicide isoproturon reduced GST activity in Z. mays leaves, indicating that certain herbicides function as GST inhibitors (Alla, Hassan, & El-Bastawisy, 2008). 587

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 592 593 of Triticum tauschii seedlings (Zhang, Xu, Lambert, & Riechers, 2007). However, it should be 594 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 595 596 protein abundance of certain phi and lambda GSTs in the weed Alopecurus myosuroides treated with the graminicidal herbicide fenoxaprop ethyl (Cummins, Bryant, & Edwards, 2009). In 597 addition, the gene encoding GSTU26 was transcriptionally induced upon exposure to the 598 599 chloroacetanilide herbicides alachlor and metolachlor and the safener benoxacor in A. thaliana seedlings. In contrast, expression levels of GSTF9 were not affected under these conditions, 600 indicating that specific GST isoforms are responsive to specific safeners and/or herbicides 601 (Nutricati, Miceli, Blando, & De Bellis, 2006). Similarly, different safeners were shown to 602 transcriptionally induce several phi and theta class GSTs in A. thaliana. Nonetheless, this response 603 604 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 605 species (DeRidder, Dixon, Beussman, Edwards, & Goldsbrough, 2002). 606

Furthermore, it should be noted that GSTs can bioactivate instead of detoxify certain organic compounds. Indeed, two examples of toxic GS-conjugates in plants refer to GST-mediated isomerisation 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, this observation is likely related to the involvement of GSTs in detoxifying products resulting from oxidative damage to cellular macromolecules. Indeed, exposure to Cd, Cu, cobalt (Co), Hg, Ni and Zn significantly increased GST activity in root tips of *H. vulgare*. Interestingly,

this response was also induced by salt, cold, drought and H₂O₂ exposure (Halušková, 615 616 Valentovičová, Huttová, Mistrík, & Tamás, 2009). Furthermore, Cd was shown to induce GST activity in roots and leaves of *Ricinus communis*. This Cd-induced increase in GST activity was 617 also observed in *B. napus* roots. As Cd exposure significantly increased MDA concentrations in 618 619 both plant species, the induction of GST activity could be targeted towards detoxifying lipid peroxidation products (Bauddh, Kumar, Srivastava, Singh, & Tripathi, 2016). In addition, Kumar 620 et al. (2013) reported that gene expression levels of GSTL1 and GSTL2 were increased by As, Cd 621 and Pb exposure in O. sativa seedlings, while they were not affected by Cr. In contrast, GSTL3 622 expression was induced by As and Pb, but not by Cd. Taken together, these data indicate that metal-623 induced transcriptional responses of GSTs depend on the metal and GST isoform under study 624 (Kumar et al., 2013; Lyubenova & Schroder, 2011). 625

626

627 *3.2.3 <u>Glutathione in antioxidative defence</u>*

Glutathione, as the most abundant essential non-enzymatic antioxidant in plant cells, contributes 628 to antioxidative defence in several ways. First of all, it can directly react with singlet oxygen $({}^{1}O_{2})$, 629 H₂O₂ and hydroxyl radicals ('OH) (Gill & Tuteja, 2010b). In addition, GSH plays an important role 630 631 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), 632 633 simultaneously oxidising 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). 634 Furthermore, GSH also contributes to antioxidative defence as an electron donor for glutathione 635 peroxidase (GPX), catalysing the reduction of H₂O₂, organic peroxides and lipid peroxides (Anjum 636

et al., 2012; Gill & Tuteja, 2010b). In addition, it reduces glutaredoxins (GRXs), which are
involved in several processes including the regeneration of antioxidative enzymes (Rouhier, 2010).
In all reactions described above, 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 percent of the
cellular GSH pool is in its reduced form under control conditions. Under 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 play a crucial role in redox
regulation (Noctor et al., 2012). However, the relevance of the GSH/GSSG redox potential as
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). 650 651 For example, Dixit et al. (2016) demonstrated that exposure to 25 and 50 μ M As significantly 652 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 653 654 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 655 656 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 657 in the growth medium, while GSSG levels decreased with increasing sulphur levels. These data 658 659 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 660 661 a more reduced state in leaves of A. thaliana exposed to Zn concentrations ranging from 100 to 500 µM, despite a significant decrease of GR activity. However, the increased GSH/GSSG ratio 662 could be explained by an increased GSH synthesis, as Zn significantly increased total GSH 663 664 equivalents and free GSH levels. In contrast, Zn slightly decreased the GSH/GSSG ratio in roots, 665 possibly as a result of the observed decrease in root GR activity. Furthermore, PC synthesis was strongly induced, thereby decreasing free GSH levels available for antioxidative defence reactions 666 in roots of Zn-exposed plants (Remans et al., 2012). In addition, Cuypers et al. (2011) reported that 667 both Cd and Cu exposure shifted the GSH redox state in A. thaliana roots towards a more oxidised 668 669 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). Furthermore, 670 Nocito et al. (2006) reported that Cd exposure caused significantly decreased total GSH levels in 671 672 roots of Z. mays. While reduced GSH levels strongly diminished, GSSG concentrations significantly increased in roots of Cd-exposed plants, thereby shifting the cellular redox balance 673 towards a more oxidised state. Zinc exposure, in contrast, did not alter the GSH/GSSG ratio, as it 674 675 increased both GSH and GSSG levels to the same extent. Again, exposure to Cu caused the most 676 dramatic effects, as it decreased the GSH/GSSG ratio by more than 20-fold (Nocito et al., 2006). 677 Taken together, 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 678 exposure duration and the plant species and organ considered. 679

As mentioned above, 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. Indeed,

exposure to metazachlor for 14 days increased the activities of APX and GR in leaves of *B. napus*, 683 684 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 oxidised form. In 685 addition, activities of other antioxidative enzymes including SOD and CAT were also induced by 686 exposure to metazachlor (Vercampt et al., 2016). Similarly, exposure to the recommended field 687 dose of metribuzin increased GSSG levels and slightly decreased the GSH/GSSG ratio in leaves of 688 Z. mays, possibly as a result of the observed decrease in GR activity. In contrast, treatment with 689 the recommended field dose of pretilachlor increased GR activity and the GSH/GSSG ratio. These 690 results possibly explain the fact that the induction of H₂O₂ levels and lipid peroxidation was more 691 692 pronounced in plants exposed to metribuzin as compared to pretilachlor. Taken together, these data indicate that herbicide-induced antioxidative plant responses depend on the specific herbicide 693 694 applied (Alla, Badawi, Hassan, El-Bastawisy, & Badran, 2008).

695

696 4 Thiols play an important role in cellular regulation

697 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) catalyses the formation of the thioether cystathionine from its 704 705 substrates cysteine and O-homophosphoserine (OPH). Subsequently, cystathionine β -lyase (CBL) 706 cleaves cystathionine to homocysteine in a β -cleavage reaction. These two steps exclusively take 707 place in plastids and are together referred to as the transsulphuration pathway (Droux, 2004; Wirtz 708 & Droux, 2005). In the third and final step of methionine synthesis, methionine is formed through 709 the methylation of homocysteine, which is catalysed by a vitamin B12-independent methionine synthase (MS), using methyltetrahydrofolate as the methyl donor (Fig. 1) (Takahashi et al., 2011). 710 711 This enzyme is present in both plastids and the cytosol. Whereas the plastidal MS isoform is only involved in *de novo* methionine synthesis, the cytosolic isoform is suggested to also mediate the 712 713 recycling of homocysteine resulting from the hydrolysis of S-adenosylhomocysteine (Gigolashvili 714 & Kopriva, 2014; Wirtz & Droux, 2005).

More than 80% of the synthesised 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 catalysed by SAM synthetase (SAMS) (Fig. 1). Subsequently, SAM serves as methyl donor in a broad array of methylation reactions catalysed by methyltransferases. Furthermore, SAM is also the precursor for the synthesis of ethylene, polyamines, 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 post-transcriptionally regulated by SAM (Amir, 2010). When SAM is present, it 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).

Furthermore, control of methionine production also occurs at the level of OPH, which is the last common intermediate for methionine and threonine biosynthesis. Although CGS and threonine synthase (TS) both 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, also methionine levels can be affected in metal-exposed plants. Indeed, 737 738 exposure to $120 \,\mu$ M Ni was demonstrated to increase methionine concentrations in roots and shoots of Matricaria chamomilla (Kovacik, Klejdus, Hedbavny, & Backor, 2009). Furthermore, exposure 739 740 to different sublethal Cd concentrations for 24 and 72 hours significantly increased methionine 741 concentrations in both roots and leaves of A. thaliana (Keunen et al., 2016a). In addition, MS 742 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 743 744 (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 745 746 contaminants can affect methionine and ethylene biosynthesis pathways in plants (Ge et al., 2009). 747 In contrast, Barkla et al. (2014) reported that Zn decreased MS abundance in leaves of A. thaliana. In addition, serine hydroxymethyltransferase levels were also reduced in Zn-exposed plants. This 748 749 enzyme catalyses 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 750 751 possibly results in a decreased methionine concentration. The authors hypothesise that this 752 mechanism protects Zn-stressed cells by attenuating the initiation of protein translation, thereby preventing the synthesis of misfolded proteins (Barkla et al., 2014). Furthermore, a Zn-induced 753 754 decrease of methionine levels was also observed in shoots of N. tabacum (Pavlíková et al., 2014). 755 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 756 757 for cysteine and other stress-responsive amino acids such as proline, glycine and glutamate. 758 Furthermore, clear correlations were reported between the levels of these amino acids, the extent 759 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). 760

761 Further evidence supporting a role for methionine in plant responses to metal stress is again derived 762 from comparing metal-tolerant and sensitive species. Begum et al. (2016) reported that As exposure 763 significantly increased methionine levels in a tolerant O. sativa genotype, whereas they were not 764 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). 765 Similarly, Liang et al. (2014) demonstrated that Cd increased root methionine levels in a 766 767 hyperaccumulating S. alfredii ecotype, while this response was absent in its non-768 hyperaccumulating counterpart. Furthermore, SAMS expression levels were only significantly 769 enhanced by Cd exposure in roots and shoots of the hyperaccumulator plants. Taken together, these 770 data point towards a role for SAM in mediating Cd tolerance in S. alfredii (Liang et al., 2014). Furthermore, MS and SAMS protein abundance were significantly increased by Cu exposure in 771 772 roots of a metallicolous population of Agrostis capillaris, while they were not affected in a non-
metallicolous population, further supporting a role for methionine and SAM in plant metaltolerance (Hego et al., 2014).

775 Whereas the effects of metals on methionine and SAM biosynthesis are relatively well studied, 776 information regarding the influence of organic contaminants on these processes is scarce and 777 therefore constitutes an interesting topic for future research. In this regard, evidence has been 778 presented that SAM-methyltransferase is involved in the detoxification of the herbicide fluorodifen 779 in *Picea*. The fluorodifen metabolite 2-nitro-4-trifluoromethyl-thiophenol was rapidly converted 780 into 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, 781 782 & Schroder, 1993). A similar pathway was demonstrated for the metabolism of 783 pentachloronitrobenzene in Allium cepa (Lamoureux & Rusness, 1980).

784

785 4.2 Ethylene: hormonal regulation

As mentioned above, thiols represent important precursors in a large number of cellular processes and regulatory networks. As an activated form of methionine, SAM is a key player in the synthesis of ethylene and polyamines (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 utilising 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*, from 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).

799 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 800 dioxygenases, ACO requires Fe²⁺ as cofactor and bicarbonate as activator (Zhang, Ren, Clifton, & 801 802 Schofield, 2004). Information on its cellular localisation remains unclear: it could be localised either in the cytosol (Chung, Chou, Kuang, Charng, & Yang, 2002; Hudgins, Ralph, Franceschi, & 803 Bohlmann, 2006) or the plasma membrane (Ramassamy, Olmos, Bouzayen, Pech, & Latche, 1998; 804 805 Rombaldi et al., 1994). Even though ACS is considered the rate-limiting step of ethylene 806 biosynthesis in plants, there are reports that point into the direction of ACO limiting ethylene 807 synthesis under certain conditions, e.g. post-climacteric fruit ripening in tomato (Van de Poel & 808 Van Der Straeten, 2014). In plants, ethylene synthesis is regulated by a variety of internal and 809 external signals, often with ACS as main target (for a review, see Van de Poel and Van Der Straeten 810 (2014) and Keunen et al. (2016b)).

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 et al., 2016b). While this was shown for several species under short time exposure conditions, longer Cd exposure decreased ethylene concentrations in *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. In addition, more and more reports suggest an implication of ethylene signalling in plant adaptation and tolerance to metal stress (Keunen et al., 2016b; 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, Kumerova et al. (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. In addition, seed germination was significantly inhibited by this compound in both species (Kummerova et al., 2012).

827

828 4.3 Polyamines: hormone-like signalling compounds

Next to ethylene synthesis, SAM is also a precursor for the synthesis of polyamines, representing 829 830 a group of low-molecular-weight polycationic amines ubiquitous in all living organisms (Liu, 831 Wang, Wu, Gong, & Moriguchi, 2015b). In plants, the positively charged polyamines are either 832 bound to negatively charged molecules or conjugated to small molecules and proteins but also 833 occur as free forms (Walters, 2003). Polyamines are involved in a large number of cellular 834 functions and regulatory processes, including the stabilisation of proteins and other biomolecules, the regulation of cell division, growth and differentiation or senescence as well as general adaptive 835 stress responses (Bouchereau, Aziz, Larher, & Martin-Tanguy, 1999; Groppa & Benavides, 2008; 836 837 Walters, 2003).

Putrescine (Put), spermidine (Spd) and spermine (Spm) are the three major polyamines and
therefore most studied in plants. They can be present in free, soluble conjugated and insoluble

bound forms. In plants, polyamine synthesis is well studied and based on the two precursors 840 841 L-ornithine and L-arginine from which Put is generated by the catalytic actions of ornithine 842 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 a first step, SAM is 843 844 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. 845 Spermidine is then further converted into Spm by Spm synthase, again using dcSAM as an 846 aminopropyl donor (Fig. 1) (Liu et al. 2015). From this reaction, MTA is released and feeded back 847 into the SAM cycle where SAM is synthesised from methionine by SAM synthase (SAMS) (Sauter 848 849 et al., 2013).

Apart from *de novo* synthesis of polyamines, it is worth mentioning that there is catabolism of these
molecules, involving both Cu-containing diamine oxidases (CuAOs) as well as FAD-dependent
polyamine oxidases (PAOs) (Cona, Rea, Angelini, Federico, & Tavladoraki, 2006; Liu et al.,
2015b).

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

As indicated in Fig. 1, SAM represents a direct link between ethylene and polyamine synthesis, allowing plants to directly switch between both pathways. Interestingly, polyamines and ethylene display counteracting functions in plants. Whereas ethylene is known to induce senescence, polyamines play the opposite role by decelerating chlorophyll loss and cell membrane degradation as well as inducing protease and RNase activity (Kusano, Berberich, Tateda, & Takahashi, 2008).
This is due to their ability to neutralise acids, their antioxidative properties as well as their potential
to stabilise cell walls and membranes (Gill & Tuteja, 2010a).

Different reports indicate a role for polyamines in plant metal stress responses. For example, 866 exposure to Cu and Cd led to an increase in ADC and ODC activity in T. aestivum leaves. This 867 effect was stronger under Cd exposure, where Put levels were elevated up to 3-fold as compared 868 869 to control levels. Spermidine concentration was not affected by any of the metals, whereas Spm levels were significantly reduced. Interestingly, externally applied Spm reduced the formation of 870 H₂O₂ and led to a reduction of TBARS levels as well. A potential antioxidative function of Spm 871 could be concluded from these results, although the actual mechanism of protection is still unclear 872 873 (Groppa, Tomaro, & Benavides, 2007).

874 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 polyamine metabolism is affected by these 875 xenobiotic compounds. Nevertheless, Burritt (2008) could link phenanthrene exposure to the 876 877 synthesis of polyamines. Phenanthrene is a polycyclic and highly toxic aromatic hydrocarbon that 878 is often found in aquatic environments. Whereas data on its effect on aquatic macrophytes are scarce, polycyclic aromatic hydrocarbons often induce oxidative stress in plants. The involvement 879 880 of polyamines 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 881 882 which the plants responded with an increase in polyamine synthesis linked to elevated ADC and SAMDC activity. Chemical inhibition of these enzymes caused an inhibition of plant recovery, 883 while externally applied polyamines could reduce the negative effects caused by phenanthrene 884 885 exposure (Burritt, 2008). More recent studies suggest the application of polyamines as priming agents prior to all sorts of abiotic stress, also including metal exposure (Savvides, Ali, Tester, &
Fotopoulos, 2016). This opens the window to more extensive research on the involvement of
polyamines in plant responses to metals as well as organic pollutants.

889

890 5 Interaction between detoxification and signal transduction pathways

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

898 [Insert Figure 2 here]

For example, cross-talk exists between ethylene and other players involved in plant responses to 899 900 metal stress, as recently reviewed by Keunen et al. (2016b). Indeed, several studies demonstrate a 901 role for ethylene in the oxidative burst induced by a broad range of stress conditions. Cao et al. (2006) demonstrated that paraquat-induced increases in O_2^{-1} and H_2O_2 levels were less pronounced 902 903 in an *ethylene insensitive 2-1 (ein2-1)* mutant as compared to those in WT A. *thaliana* plants. As a 904 consequence, 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 (Zhang, He, Zhao, Huang, 905 & Hao, 2014b). In addition, blocking of ethylene biosynthesis and/or signalling resulted in a 906

reduced extent of H₂O₂ production in camptothecin-exposed *Lycopersicon esculentum* suspension 907 908 cells (de Jong, Yakimova, Kapchina, & Woltering, 2002) and Cd-exposed Phaseolus coccineus 909 roots (Maksymiec, 2011). Furthermore, inhibition of ethylene biosynthesis limited Cd-induced O2⁻⁻ accumulation in root hair tips of *B. napus*, suggesting that ethylene is an upstream regulator of O_2^{-1} 910 911 generation (Sun & Guo, 2013). The effect of ethylene on O_2^{-} production is possibly mediated by its interaction with ROS-producing NADPH oxidases, also referred to as respiratory burst oxidase 912 homologues (RBOHs). This is confirmed by the observation that H₂O₂ production induced by the 913 914 ethylene releasing compound ethephon in *I. batatas* was limited by treatment with the NADPH inhibitor diphenyleneiodonium (Chen, Huang, Huang, Chow, & Lin, 2013). This relationship 915 916 between ethylene and NADPH oxidases is also supported under metal exposure conditions, as Hg-917 induced increases in apoplastic H₂O₂ accumulation and NADPH oxidase activity in apical root segments of Medicago sativa were reduced by treatment with an ethylene signalling inhibitor 918 (Montero-Palmero, Martin-Barranco, Escobar, & Hernandez, 2014). Furthermore, Keunen et al. 919 (2015) reported that the Cd-induced RBOHC upregulation in several A. thaliana ethylene 920 921 biosynthesis and signal transduction mutants was less pronounced as compared to that in WT 922 plants.

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 non-enzymatic antioxidants such as AsA and α -tocopherol (Keunen et al., 2016b). In the context of this review, the interaction between ethylene and GSH is of particular interest. The crosstalk between these two compounds is probably related to the link of ethylene biosynthesis and signalling with sulphate assimilation (Fig. 2), as was recently reviewed by Iqbal et al. (2013) and Wawrzynska et al. (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). 930 931 Nazar et al. (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, 932 suggesting a role for ethylene in the sulphur-induced salt stress alleviation (Nazar et al., 2014). 933 934 Furthermore, several genes involved in ethylene signalling were strongly upregulated in sulphate-935 treated Vitis vinifera (Giraud, Ivanova, Gordon, Whelan, & Considine, 2012). In addition to sulphur 936 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). 937

Interestingly, the interplay between ethylene and sulphate assimilation also functions in the other 938 direction, with ethylene levels affecting several proteins of the sulphate assimilation pathway (Fig. 939 2). Indeed, it was shown that ethephon significantly induced ATPS activity and sulphur content in 940 941 B. juncea (Iqbal, Khan, Nazar, & da Silva, 2012). Furthermore, treatment of A. thaliana with 200 942 μ M ACC was shown to induce APR at the transcriptional and activity level (Koprivova, North, & 943 Kopriva, 2008). As GSH synthesis depends on sulphur availability for the production of its 944 precursor cysteine, ethylene possibly modulates the sulphate assimilation pathway in order to meet 945 the increasing demand for GSH during stress conditions (Keunen et al., 2016b).

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). Furthermore, the ethephon-induced increased ROS production in sweet potato was attenuated when plants were treated with exogenous GSH (Chen et al., 2013). In addition, expression levels of ethylene-related genes are often affected in transgenic plants with altered GSH levels (Keunen et al., 2016b). 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 953 954 to WT plants. In contrast, ERF2 expression was significantly downregulated in the 955 A. thaliana cad2-1 mutant, which is characterised by a milder GSH deficiency (Han, Mhamdi, 956 Chaouch, & Noctor, 2013). Furthermore, transgenic tobacco plants with an enhanced GSH content 957 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 958 A. thaliana plants with elevated GSH levels displayed increased ACS2, ACS6 and ACO1 959 960 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-961 962 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. 963 Interestingly, application of exogenous GSH also increased the tolerance of WT A. thaliana plants 964 965 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 966 stress factors by an ethylene-dependent mechanism (Datta et al., 2015). 967

968 Interestingly, the relationship between ethylene and GSH is also supported under metal exposure 969 conditions (Keunen et al., 2016b). Indeed, ethephon application was shown to increase GSH 970 concentrations in B. juncea exposed to Cd (Masood, Iqbal, & Khan, 2012), Ni and Zn (Khan & 971 Khan, 2014). Furthermore, Schellingen et al. (2015) reported that the Cd-induced upregulation of 972 genes involved in GSH biosynthesis was significantly weaker in an acs2-1/6-1 knockout mutant as 973 compared to WT A. thaliana plants, suggesting that ethylene biosynthesis is essential for the 974 induction of efficient GSH-dependent defence responses upon Cd exposure (Schellingen et al., 2015). Similarly, the crucial role of EIN2 in Pb tolerance in A. thaliana was also demonstrated to 975

be partially related to its stimulating effect on GSH levels (Cao et al., 2009). It should be noted that
the interaction between ethylene and GSH under metal exposure conditions also functions in the
other direction, with GSH levels affecting ethylene signalling. Indeed, Hasan et al. (2016) reported
that the Cd-induced upregulation of *ERF1* and *ERF2* was more pronounced in plants supplied with
5 mM GSH, while it was weaker in plants treated with the GSH biosynthesis inhibitor buthionine
sulphoximine.

982 In addition to ethylene, polyamines are also subject to cross-talk with the antioxidative defence system during stress conditions. Addition of Spd to Solanum lycopersicum exposed to chilling 983 stress, for example, strongly enhanced the chilling-induced increase in GSH and AsA levels. 984 Furthermore, it significantly increased the GSH/GSSG and AsA/DHA ratios, possibly as a result 985 986 of the observed induction of enzymes involved in the AsA-GSH cycle at the transcriptional and/or 987 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 988 control plants. These data possibly explain the observed reduction of chilling-induced O_2^{-} and 989 990 H₂O₂ production by addition of exogenous Spd (Diao, Song, & Qi, 2015). Similarly, positive effects of polyamine treatment on the antioxidative defence system were also reported in salt-991 exposed O. sativa (Jain, Vart, Verma, & Malhotra, 2015) and Cucumis sativus (Shu, Yuan, Guo, 992 993 Sun, & Yuan, 2013) and Glycine max. subjected to osmotic stress (Radhakrishnan & Lee, 2013).

Polyamines were also shown to positively affect several components of the antioxidative defence system in metal-exposed plants. Indeed, Spd treatment was shown to further enhance the Crinduced increase in GSH levels in *Rhapus sativus*. Furthermore, Spd significantly alleviated Crinduced lipid peroxidation and H₂O₂ concentrations. In contrast, it counteracted the increase in CAT activity caused by Cr exposure. Overall, exogenous Spd increased plant tolerance to Cr, as

999 indicated by its positive effects on root and shoot length and fresh weight of Cr-exposed plants 1000 (Choudhary, Kanwar, Bhardwaj, Yu, & Tran, 2012). Similarly, Rady and Hemida (2015) reported 1001 that addition of either Spd or Spm counteracted the negative effects of Cd exposure on several 1002 growth parameters of *T. aestivum* plants. This effect was likely related to the fact that polyamine 1003 application attenuated Cd-induced effects on leaf GSH levels and SOD, CAT and APX activity. As a result, Cd-induced lipid peroxidation and H₂O₂ production were reduced in leaves of 1004 polyamine-treated plants. However, these data should be interpreted with caution, as the 1005 application of Spd and Spm also significantly reduced shoot Cd concentrations potentially 1006 explaining the observed attenuation of metal stress (Rady & Hemida, 2015). 1007

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

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1015 6 Concluding remarks and future perspectives

1016 The sulphur-containing amino acids cysteine and methionine play crucial roles in plant responses 1017 to metals and organic contaminants through their incorporation into the primary metabolites GSH 1018 and SAM. Whereas GSH is primarily involved in chelation and detoxification mechanisms, SAM 1019 mainly contributes to signal transduction reactions mediated by ethylene and polyamines.

Significant cross-talk exists between these detoxification and signal transduction pathways, which 1020 1021 is explained by the fact that cysteine is a precursor for both GSH and methionine synthesis. 1022 Furthermore, ROS appear to play an important role in mediating the interaction between both 1023 pathways. Whereas the effects of metal exposure on plant detoxification and signal transduction 1024 pathways are relatively well described, information regarding the influence of organic contaminants is rather scarce and mostly limited to herbicides and safeners, indicating a need for 1025 further research. Elucidating plant responses to metals and organic compounds can significantly 1026 1027 contribute to optimising phytoremediation strategies for the clean-up of contaminated soils. In this strategy, special attention should be given to sulphur availability in the environment as it affects 1028 sulphate assimilation and downstream detoxification and regulation pathways that are important 1029 1030 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 1031 1032 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 1033 could be achieved by molecular approaches, fostering plant-microbe interactions or the use of 1034 1035 sustainable agrochemical amendments.

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8 Tables

Table 1: Natural functions of GSTs in plants.

Postulated natural role for GSTs	Reference
	Diesperger & Sandermann, 1979
Conjugation of endogenous metabolites	Edwards & Dixon, 1991
	Dean et al., 1995
Conjugation of DNA degradation products	Morgenstern et al., 1985
	Lamoureux & Frear, 1987
Conjugation of phytohormones	Meyer et al., 1991
(auxin, ethylene, gibberellic acid)	Takahashi & Nagata, 1992
	Zettl et al., 1994
	Mannervik & Danielson, 1988
Detoxification of lipid hydroperoxides	Marrs, 1996
	Sommer & Böger, 1999
Transport of (thio-)phenols,	Singh & Shaw, 1988
chlorophyllin and anthocyans	Martinoia et al., 1993
Regulation of GSH pool	Lamoureux & Rusness, 1989
Detoxification of fungal toxins	Dudler et al., 1991
and pathogen defence	Mauch & Dudler, 1993
Increase of drought tolerance	Dhindsa, 1991

Antioxidative protective protein	Levine et al., 1994
Conjugation and transport of phytoalexins and similar compounds	Li et al., 1997 Marrs et al., 1995
Regulation of UV-induced genes	Loyall et al., 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 Dixon & Edwards, 2010
Isomerisation and peroxidation	Cummins et al., 2011
Deglutathionation of GS-conjugates	Lallement et al., 2014

Fig. 1: Biosynthesis pathway of sulphur-derived cellular compounds and their involvement 1805 in metal scavenging, xenobiotic detoxification, antioxidative defence and signal transduction. 1806 Abbreviations: acetyl-CoA, acetyl-coenzyme A; ACC, 1-aminocyclopropane-1-carboxylic acid; 1807 ACO, ACC oxidase; ACS, ACC synthase; APK, APS kinase; APR, APS reductase; APS, 5'-1808 phosphosulphate; APX, ascorbate peroxidase; AsA, ascorbate; ATPS, ATP sulphurylase; CBL, 1809 cystathionine β -lyase; CGS, cystathionine γ -synthase; Cys, cysteine; dcSAM, decarboxylated 1810 SAM; DHA, dehydroascorbate; DHAR, DHA reductase; γ -EC, γ -glutamylcysteine; γ -ECS, γ -EC 1811 1812 synthetase; γ -Glu, γ -glutamate; Gly, glycine; GR, glutathione reductase; GS-conjugate, glutathione S-conjugate; GSH, glutathione; GSH-S, glutathione synthetase; GSSG, glutathione disulphide; 1813 GST, glutathione transferase; H₂O, water; H₂O₂, hydrogen peroxide; HCys, homocysteine; L-Ser, 1814 L-serine; Met, methionine; MS, methionine synthase; MT, metallothionein; OAS, O-acetylserine; 1815 OASTL, OAS (thiol) lyase; OPH, O-homophosphoserine; PAPS, 3'-phosphoadenosine 5'-1816 phosphosulphate; PC, phytochelatin; PCS, phytochelatin synthase; Put, putrescine; S²⁻, sulphide; 1817 SAM, S-adenosylmethionine; SAMDC, SAM decarboxylase; SAMS, SAM synthetase; SAT, 1818 serine acetyl transferase; SiR, sulphite reductase; SO_3^{2-} , sulphite; SO_4^{2-} , sulphate; Spd, spermidine; 1819 Spd synthase, spermidine synthase; Spm, spermine; Spm synthase, spermine synthase; SULTR, 1820 1821 sulphate transporter.

1822

Fig. 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. Abbreviations: Cys, cysteine; GSH,
 glutathione; GSSG, glutathione disulphide; GST, glutathione transferase; Met, methionine; PC,
- 1830 phytochelatin; ROS, reactive oxygen species; SAM, S-adenosylmethionine.

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