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Review

Low-molecular-weight thiols in plants: Functional and analytical implications

Micaela Pivato, Marta Fabrega-Prats, Antonio Masi *

DAFNAE, University of Padova, Viale Università 16, 35020 Legnaro, PD, Italy

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ABSTRACT

Low-molecular-weight (LMW) thiols are a class of highly reactive compounds massively involved in the maintenance of cellular redox homeostasis. They are implicated in plant responses to almost all stress factors, as well as in the regulation of cellular metabolism. The most studied LMW thiols are glutathione and its biosynthetically related compounds (cysteine, γ-glutamylcysteine, cysteinylglycine, and phytochelatins). Other LMW thiols are described in the literature, such as thiocysteine, cysteamine, homocysteine, lipoic acid, and many species-specific volatile thiols. Here, we review the known LMW thiols in plants, briefly describing their physico-chemical properties, their relevance in post-translational protein modification, and recently-developed thiol detection methods. Current research points to a huge thiol biodiversity in plants and many species-specific and organ-specific thiols remain to be identified. Recent advances in technology should help researchers in this very challenging task, helping us to decipher the roles of thiols in plant metabolism.

Introduction

Reduced sulfur is contained in several biomolecules of living organisms, especially in proteins such as methylated in methionine (Met)[1] and as thiol in cysteine (Cys), but there are probably hundreds of non-protein molecules constituting the sulfur metabolome [1]. In the past, it was assumed that about 2% of the organic reduced sulfur in plants occurs in the form of non-protein, low-molecular-weight (LMW) thiols [2]. The thiol moiety is one of the strongest nucleophilic groups in the cell. It is involved in a number of chemical reactions that give thiol-containing molecules a primary role in cellular redox homeostasis, in controlling enzyme activity and detoxifying reactive oxygen/nitrogen species and xenobiotics, as well as in the formation of disulfide bonds needed to define the structural characteristics and regulatory properties of proteins [3]. The main cellular LMW thiols are Cys and glutathione (GSH). Cys occupies a key position on numerous metabolic pathways, and it is generally found at low concentrations because it is rapidly converted into other compounds or incorporated in proteins [4]. GSH is the most abundant and best described LMW thiol in both plants and animals because of its importance in redox and regulatory functions.

This review focuses on describing the known LMW thiols in plants, including a number of less well-known compounds that are generally neglected, but play a significant part in plant metabolism. The distinction between plant and animal thiols is necessary for two reasons: first, the metabolic pathways differ considerably, given that animals are unable to assimilate inorganic sulfur and produce Cys from Met, as happens in plants; and second, there are different thiols in the two kingdoms (for example, phytochelatins are a group of LMW thiols peculiar to plants). This work therefore focuses only on plant LMW thiols, although they have many chemical characteristics and functional properties in common with animal thiols.

Plant sulfur metabolism

Sulfur is an element essential to plant primary metabolism, as a structural component of proteins and lipids, some vitamins and regulatory molecules, antioxidants, metal-binding molecules and cofactors/coenzymes [4]. Plants take up inorganic sulfur mainly from soil in the form of anionic sulfate (SO_3^{2-}), and specific transporters actively carry it to their leaves [5]. Anionic sulfate can become a vacuolar sap component as it is, or be fixed to organic molecules after reduction reactions involving a class of ATP sulfurylases and APS reductases [6]. Sulfite (SO_3^-) can be added to an

* Corresponding author.
E-mail address: antonio.masi@unipd.it (A. Masi).

[1] *Abbreviations used:* Cys, cysteine; Cys-Gly, cysteinylglycine; DHLA, dihydrolipoic acid; γ-Glu-Cys, γ-glutamylcysteine; Grx, glutaredoxin; GSH, glutathione; GSSG, glutathione disulfide; Hcys, homocysteine; hGSH, homoglutathione; LA, lipoic acid; LMW, low-molecular-weight; Met, methionine; PC, phytochelatins; PTM, post-translational modification; SAM, S-adenosylmethionine; Trx, thioredoxins.

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organic molecule by means of the sulfation reaction, or it can be further reduced to sulfide (S^{2-}), and subsequently fixed to Cys, and thus enter a variety of synthesis pathways [7]. SO_4^{2-} is reduced to sulfide (S^{2-}) in the chloroplast as a result of the addition of eight electrons derived from photosynthesis in a multistep pathway that requires one ATP [5]. On the other hand, the sulfation reaction is catalyzed mainly in the cytosol by specific sulfotransferases that covalently add the sulfonate group from 3′-phosphoadenosine 5′-phosphosulfate (known as PAPS) to a hydroxyl group of an organic molecule. Sulfotransferases are involved in the synthesis of glucosinolates, certain flavonoids and jasmonates, sulfo-glycolipids, and in tyrosine post-translational modification (PTM) [8].

Cys is the main product of sulfur assimilation and has a core role in sulfur metabolism (Fig. 1). The cellular concentration of Cys is quite low, however, because it is rapidly incorporated in proteins or converted into other compounds [4], mainly Met and GSH. Cys is also the precursor of a number of non-thiol sulfur compounds, including vitamin H (biotin), vitamin B1 (thiamine), coenzyme A, the molybdenum cofactor, certain phytoalexins, Fe–S clusters [9].

Met and Cys are the only sulfur-containing amino acids and, unlike Cys, Met does not have a thiol moiety, but is synthesized through the sequential formation of cystathionine and homocysteine (Hcys), both LMW thiols discussed in this review (see Homocysteine section). Met plays an important part in plant metabolism as a precursor of S-adenosylmethionine (SAM), a non-thiol compound participating in the synthesis of the polyamines spermidine and spermine (involved in regulating plant growth and stress responses), the metal ion chelating compounds nicotinamide and the phytosiderophores (common in higher plants), and the gaseous plant hormone ethylene [10]. SAM is also the methyl donor for a variety of macromolecules, including proteins, nucleic acids and polysaccharides [11].

GSH is considered the most important LMW thiol in plants because of its pivotal role in sulfur metabolism as the preferred molecule for storing reduced sulfur. It can move through xylem and phloem fluids, so it is involved in long-distance sulfur transport between organs. It can be rapidly converted into Cys or other compounds and it is used to protect the cell from oxidative stress, detoxify xenobiotics, and regulate protein function. It is also the precursor of phytochelatins (PCs) and other molecules involved in plant signaling and regulation (see Synthesis and degradation: glutathione, gamma-glutamylcysteine and cysteinylglycine and Phytochelatins sections).

Met and GSH, together with other sulfate compounds such as sulfolipids, are considered the cell's source of sulfur and, if necessary, they are converted by specific enzymes into Cys, to return part of sulfur metabolism.

Thiol properties

The thiol group mainly occurs in cells as an amino acid side chain moiety of Cys, the main product of plant sulfur assimilation. In addition to being a component of thiol-containing proteins and LMW thiol compounds, the amino acid Cys is a crucial metabolite for the synthesis of sulfur-containing molecules like Met, some vitamins (e.g. thiamine and biotin), lipoic acid (LA) and coenzyme A.

At physiological pH, Cys residues are protonated, but sublocally higher pH levels and polar or basic amino acids nearby can reduce Cys pK_a, deprotonating the thiol, and the resulting thiolate anion is one of the strongest biological nucleophiles in the cell [12,13]. As a result, Cys is more reactive and is involved in a series of redox chemical reactions that enable it to have both structural properties and functional activities as part of the catalytic sites of different classes of enzymes [14].

Disulfide bonds

Disulfide bonds could form both from the thiolate anion, catalyzed by specific enzymes, and spontaneously, generally through an oxidized intermediate (sulfenic acid, —SOH), [15]. Disulfide bond formation is generally induced by nucleophilic substitution, which can often involve oxidoreductive interchange mechanisms between reducing equivalents of Cys and other compounds ($FADH_2$, NADPH, GSH or Cys residues of proteins) [14,15].

Stable disulfide linkages between Cys exert fundamental structural functions in protein folding: intra-molecular disulfide bonds stabilize the protein's tertiary structure, improving rigidity (e.g.

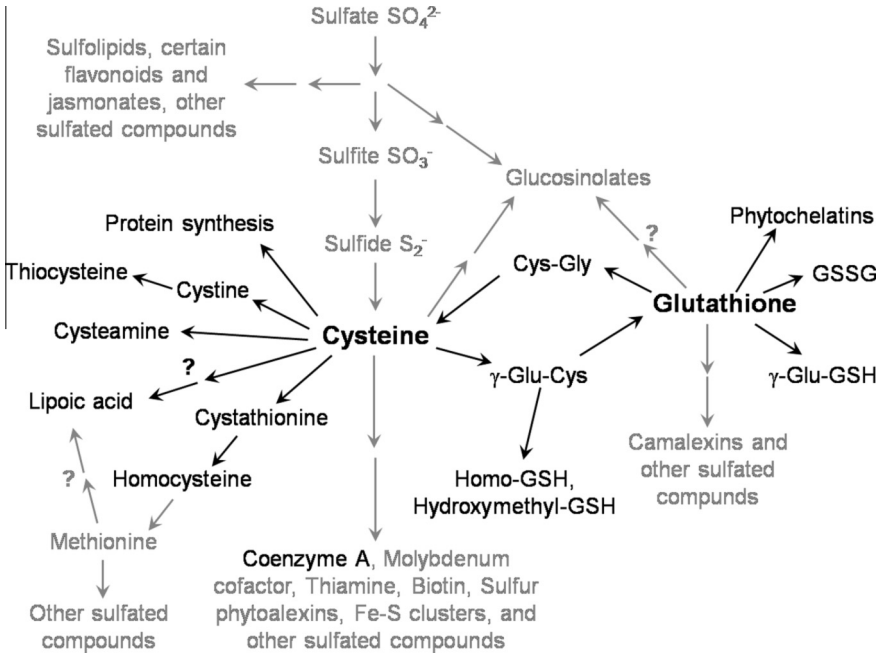


Fig. 1. Central role of Cys and GSH in sulfur-thiol metabolism; in black: thiols, in gray: non-thiol sulfur compounds.

loop formation), whereas inter-molecular disulfide bonds between different polypeptide chains support the protein's quaternary structure. For instance, the 11S legumin storage proteins, which include glycinin, are non-covalent hexamers whose monomers consist of two different subunits linked by disulfide bonds [16]. Disulfide bonds are also needed for the oligomerization of covalent dimer proteins (homo- or heterodimers), like some heat shock proteins and chaperones, or for higher-level oligomerization (e.g. transcriptional factors important to plant immunity such as the nonexpressor of pathogenesis-related protein 1, NPR1) [17].

Reversible disulfide linkages on reactive Cys (with a lower pK_a) form the basis of cellular redox maintenance and participate in regulating enzyme activity. LMW thiols spontaneously bind to proteins to protect reactive Cys from reactive oxygen species (ROS) attack under conditions of stress. Protein adducts with GSH and cysteinylglycine (Cys-Gly) could also have regulatory functions (see Protein thiolation section) [12,18].

Two complex families of regulatory enzymes, thioredoxins (Trx) and glutaredoxins (Grx), exert their function by catalyzing Cys thiol/disulfide exchanges. They enable the reduction of protein substrates, and are in turn regenerated exchanging reducing equivalents with NADPH (Trx) and GSH (Grx). A huge number of enzymes provide the substrate for their action (e.g. storage proteins, transcriptional factors, ribonucleotide reductases, etc.), so a number of cellular pathways are regulated via thiol/disulfide mechanisms, including photosynthesis, seed germination, Cys metabolism, and others [19,20].

Enzyme catalytic sites

Reactive Cys are the catalytic site of a number of enzymes involved in redox reactions, such as oxidases, peroxidases, reductases and dehydrogenases. Jacob and colleagues have thoroughly described the chemical mechanisms defining these reactions [14]. Briefly, depending on the composition of the amino acid environment at reactive sites, Cys can catalyze thiol/disulfide exchanges (as already mentioned for Trx and Grx), and also electron transfer and hydrogen atom transfer reactions. These reactions are implicated in countless processes, ranging from the maintenance of redox homeostasis to energy production.

For example, in the enzyme glyceraldehyde 3-phosphate dehydrogenase, the thiolate anion conducts a nucleophilic attack on the carbonyl group of aldehyde, forming a tetravalent thioether that readily facilitates the progress of hydride transfer. The nucleophilic attack on carbonylic carbons is also the first step in the action of lipases and proteases that contain Cys at their active site. In plants, different classes of Cys proteases are involved in biotic and abiotic stress responses, programmed cell death, and storage protein deposition and degradation [21]. The same catalytic reactions are also employed by acyl–acyl carrier thioesterases involved in the synthesis of fatty acids in plants (of primary interest because they are related to seed lipid production) [22].

Metal coordination

As a thiolate anion, Cys coordinates protein-metal binding with a number of physiological metal ions, such as Fe, Zn, Cu, as well as xenobiotic Co, Ag, Cd and Hg, but it is unable to interact with group 1 and 2 metal ions [14]. The relevance of this capability should be glaring, given that one in two known proteins are believed to contain metal cofactors [23], and the processes that involve metalloproteins include photosynthesis, respiration, signal transduction, epigenetic processes and many others. Cys-metal ion interaction can have both structural (e.g. zinc finger proteins) and functional roles (e.g. metalloproteases).

To give some examples, ferredoxins are iron-sulfur proteins that mediate electron transfer in a variety of reactions involved in a number of molecular pathways (photosynthesis, chlorophyll synthesis, and others) [24]. Metallothioneins form a family of Cys-rich proteins involved in metal transport, storage and detoxification of non-essential metals or excessive amounts of essential metals [25]. Together with PCs (see Phytochelatins section), they chelate cytosolic metals in cases of excessive heavy metal load.

Protein thiolation

PTMs on Cys residues represent the major and most significant redox alterations in plant cells. The physiological relevance of PTMs is underscored by their variety and by the reversibility of most of the chemical reactions involved. As discussed above, the reactivity of the thiol group means that Cys can undergo a number of different redox reactions and result in disulfide bonds, sulfenic acids (S—OH) and further states of oxidation to sulfinic and sulfonic acids (—SO_2H and —SO_3H), sulfhydryl moieties (S—SH), nitrosothiols (S—NO), and, less commonly, S-sulfenyl-amides (S—N) and thiosulfinates (SO—S) [26].

One of the Cys redox PTMs involves the formation of disulfide bonds with LMW thiols, known as protein thiolation. The term has often been used inappropriately as a synonym for protein glutathionylation (the formation of protein-GSH adducts) – the best-documented protein-LMW thiol linkage – but binding to Cys-Gly, called protein cysteinylglycylation, and to free Cys (protein cysteinylation) have also been reported and belong to the thiolation PTMs.

Glutathionylation occurs as a defense mechanism in response to oxidative stress, enabling the cell to protect the Cys residues particularly prone to oxidation (i.e. the "reactive" Cys, with low pK_a) from being irreversibly oxidized. It also occurs in physiological conditions as a regulatory mechanism and signaling process [12,27]. Glutathionylation modulates cellular life cycle processes (division, differentiation, programmed cell death), energy metabolism and glycolysis, protein folding and degradation, pathogen resistance, certain stages of plant development (rhizobia symbiosis, seed maturation, desiccation), and many other processes [28–32]. Protein thiol-GSH adducts form spontaneously, but can be catalyzed by Grx as well, which also has the capacity to revert the reaction to GSH and reduced Cys.

A recently-released database dedicated to glutathionylation, dbGSH [33], integrates the available datasets on experimentally verified glutathionylation sites (mapped as UniProtKB entries) and provides structural and functional analytical tools and links to the online literature. As at December 2013, the dbGSH counted more than 2200 experimentally verified S-glutathionylated proteins, most of them murine (1128) or human (1008). Only 12 proteins are reported for *Arabidopsis thaliana* and a few other plant species, despite the plethora of studies asserting the relevance of glutathionylation in plant biology. This is probably attributable to the fact that the database only contains experimentally verified glutathionylated sites and the related peptides, detectable by means of advanced mass spectrometry analyses on PTMs (see LMW thiol separation and detection techniques section), and impossible to identify using indirect methods that detect free LMW thiols after protein reduction. There will presumably be more proteomic studies on thiolation PTMs in plant organisms in the future, and it is reasonable to expect many of the glutathionylated proteins found in mammals to be modified in plants too.

Cysteinylation and cysteinylglycylation have been reported in bacteria and mammals, and the available data strongly suggest their involvement in regulating cell metabolism, like glutathionylation. In *Salmonella typhimurium*, cysteinylation occurs preferentially under infection-like conditions on the same residues where glutathionylation takes place in the basal physiological state [34]. In human plasma, linkages to Cys and Cys-Gly seem to be more abundant than glutathionylation as thiolation PTMs in globulins

and albumin [35]. To our knowledge, these modifications have yet to be reported in plants, though the existence of these PTMs cannot be ruled out.

LMW thiol separation and detection techniques

It is important to identify and quantify LMW thiols in plants to shed light on their biological function and metabolism. Several methods have been developed for this purpose, generally based on five main steps: (i) extraction,; (ii) reduction; (iii) derivatization; (iv) separation; and (v) detection. LMW thiols are found in cell as free, soluble thiols that can be reduced and oxidized (e.g. GSH and GSSG), or linked to proteins. The above steps are adapted to suit the aims of a given study, i.e. different experimental flowcharts are used if researchers are interested in profiling the total LMW thiol content, or quantifying the redox state of free thiols, or characterizing thiolation PTMs under specific cellular conditions and the amino acid position where the modifications occur (see schematic overview in Fig. 2).

Extraction

Free thiols are usually extracted in an acidic environment to protonate the —SH groups. The most often used solutions contain chlorhydric, perchloric, sulfosalicylic or metaphosphoric acid. The acidic conditions induce the precipitation of the proteins, which can then be separated from the free thiols with a simple centrifugation step. To study thiolation, the protein pellet is resuspended with detergents (e.g. SDS, Tween) or other solubilizing agents (e.g. guanidine) for further analysis.

Reduction

To be detectable, thiols must be reduced prior to any subsequent modification steps. The most frequently used reducing reagents are thiol-containing reductants such as the monothiol β-mercaptoethanol (ME) and the dithiol dithiothreitol (DTT), but they demand an additional removal step (e.g. by gel filtration) to prevent any interference with the derivatization agents. Phosphines are currently preferred in order to avoid this removal step, since they do not usually participate in further reactions. Some examples of phosphines are tris-(2-carboxyethyl)phosphine (TCEP) and tributylphosphine (TBP) [36].

Derivatization

Derivatization consists in the chemical labeling of reduced thiols with compounds that enable their detection. All these reagents prompt an irreversible thiol-disulfide exchange reaction that results in an increase in the thiol molecular mass, and most of them carry a chromogenic or fluorescent moiety for the purpose of detection using spectrophotometric techniques. For mass spectrometry (MS), a labeling step is not strictly necessary, but alkylating reagents are commonly used to distinguish reduced from oxidized thiols. Table 1 lists some commonly-used derivatization reagents.

Examples of labeling reagents for UV detection are DTNB (5,5′-dithio-bis-(2-nitrobenzoic acid)), also known as Ellman's reagent [37], 4-DPS (a similar reagent), CMPI (2-chloro-1-methylpyridinium iodide), and CMQT (2-chloro-1-methylquinolinium tetrafluoroborate) [66]. DTNB and 4-DPS are aromatic disulfide compounds, so any reducing reagent must be removed before labeling. A post-column HPLC-UV detection method for detecting LMW thiols has also been developed, based on the aggregation of gold nanoparticles functionalized with nonionic surfactant [43].

By comparison with UV detection, derivatization with fluorescent dyes is more sensitive and thiol-selective, and it can be done using a variety of reagents [67]. Monobromobimane (mBBr) has been amply used both for quantifying LMW thiols and for analyzing thiol-containing proteins [36,44]. Other fluorescent derivatives

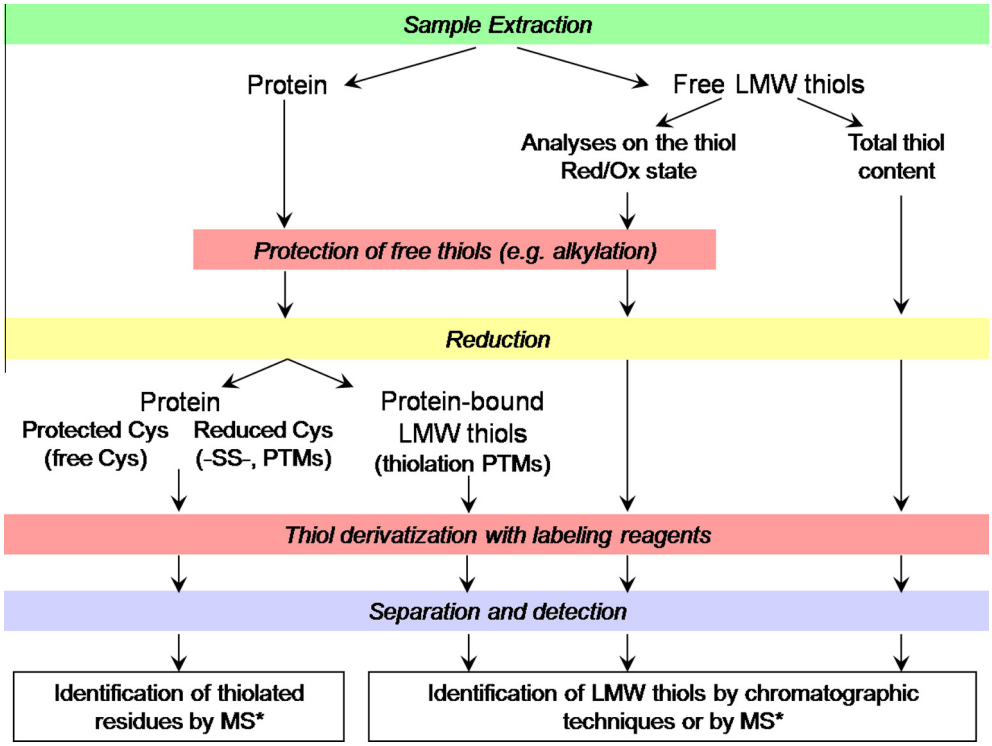
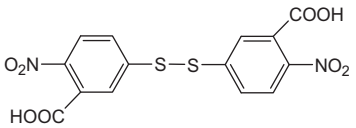
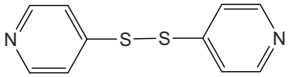
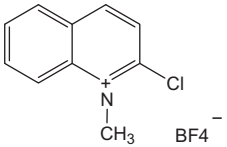
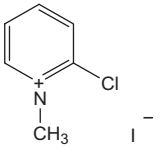
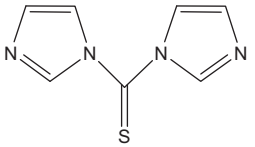
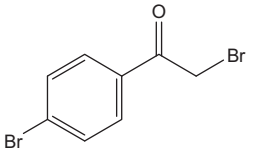
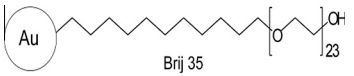
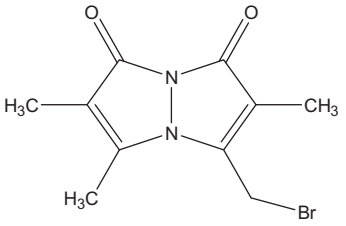


Fig. 2. Flow chart of sample preparation and analysis. Given the experimental aims, five main steps are needed to study thiolation PTMs and total thiol content, or to assess the thiol redox state (extraction, reduction, derivatization, separation and detection). * Free thiols can be identified by MS analysis (both GC- and LC–MS) without any derivatization.

Table 1
Commonly used derivatizing reagents, together with their chemical structure and references.

Category	Compound	Structure	References
UV Reagents (HPLC-UV)			
Aromatic disulfides	DTNB (5,5′-dithio-bis-(2-nitrobenzoic acid) or Ellman's reagent)		Ellman (1959) [37]
	4-DPS (4,4′-dithiodipyridine)		Grassetti and Murray (1967) [38]
Others	CMQT (2-chloro-1-methyl quinolinium tetrafluoroborate)		Bald and Glowacki (2001) [39]
	CMPI (2-chloro-1-methylpyridinium iodide)		Kaniowska et al. (1998) [40]
	TDCI (1,1′-thiocarbonyldiimidazole)		Amarnath and Amarnath (2002) [41]
	p-BPB (p-bromo phenacyl bromide)		Huang et al. (2006) [42]
	GNPs (Gold nanoparticles)		Lu et al. (2007) [43]
Fluorescent reagents (HPLC-FL)			
Bimanes	mBBr (monobromobimane)		Fahey and Newton (1987) [44]

(*continued on next page*)

Table 1 (*continued*)

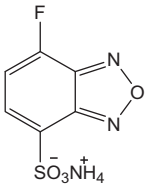
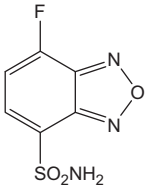
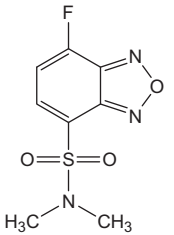
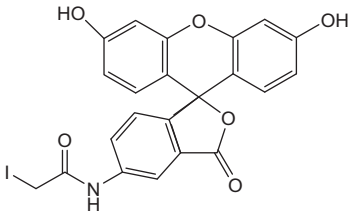
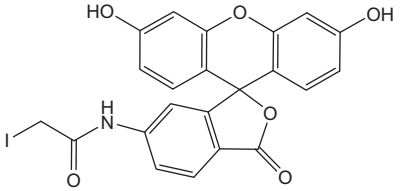
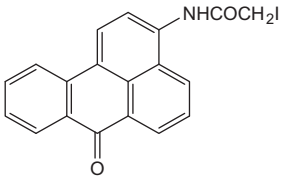
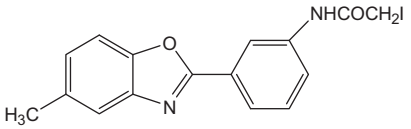
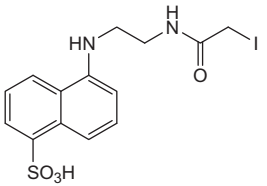
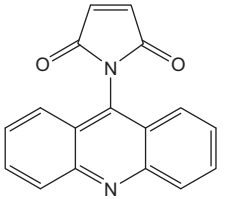
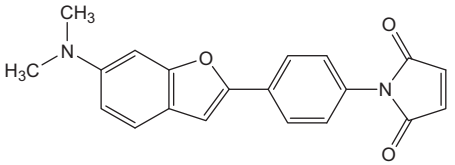
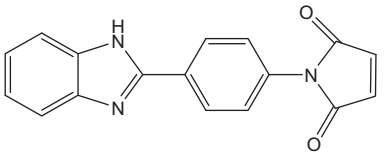
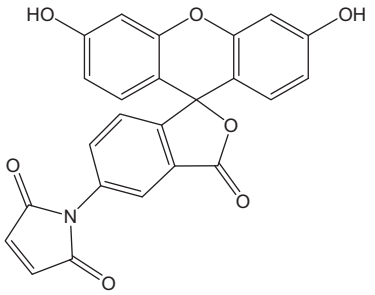
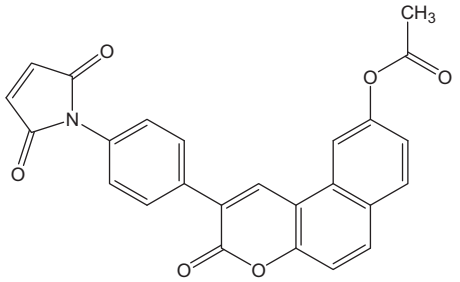
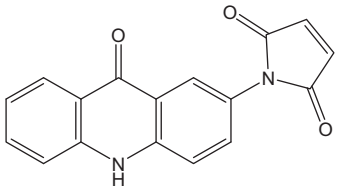
Category	Compound	Structure	References
Halogeno benzofurazans	SBD-F (ammonium 7-fluoro 2,1, 3-benzooxadiazole-4-sulfonate)		Oe et al. (1998) [45]
	ABD-F (4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole)		Toyo'oka and Imai (1984) [46]
	DBD-F (4-(N,Ndimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole)		Toyo'oka et al. (1989) [47]
Halides	5-IAF (5- iodoacetamidofluorescein)		Carru et al. (2004) [48]
	6-IAF (6- iodoacetamidofluorescein)		Causse et al. (2000) [49]
	IAB (3-iodoacetylaminobenzanthrone)		Wang et al. (2004) [50]

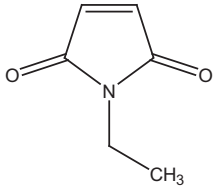
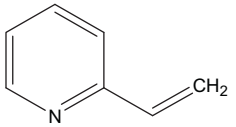
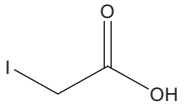
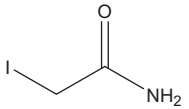
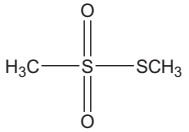
Table 1 (*continued*)

Category	Compound	Structure	References
	MIPBO (5-methyl(2-(m-iodoacetylaminophenyl)benzoxazole)		Liang et al. (2005) [51]
	1,5-I-AEDANS (5-({{2-[iodoacetyl]amino}ethyl}amino)naphthalene-1-sulfonic acid)		Clements et al. (2005) [52]
Maleimides	NAM (N-(9-acridinyl)maleimide)		Akasaka et al. (1986) [53]
	DBPM (N-(p-(2-(6-dimethylamino)benzofuranylphenyl)maleimide))		Nakashima et al. (1985) [54]
	BIPM (N-[p-(2-benzimidazolyl)phenyl]maleimide)		Kanaoka et al. (1970) [55]
	FM (fluorescein-5-maleimide)		Reddy et al. (1998) [56]

(*continued on next page*)

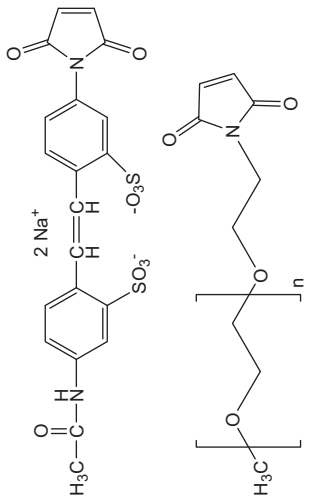
Table 1 (*continued*)

Category	Compound	Structure	References
	ThioGlo™ 3 (9-acetoxy-2-(4-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)pyeny)-3-oxo-3H-naphtho[2,1-b]pyran)		Yang and Langmuir (1991) [57]
	MIAC (N-(2-acridonyl)maleimide)		Benkova et al. (2008) [58]

Compound	Structure	References
Alkilating reagents		
NEM (*N*-ethylmaleimide)		Gregory (1955) [59]
VP (2-vinylpyridine)		Griffith (1990) [60]
IAA (iodoacetic acid)		Reed et al. (1980) [61]
IAM (iodoacetamide)		Maeda et al. (2005) [62]
MMTS (S-methyl methanethiosulfonate)		Laszlo and Mathy (1984) [63]

Joly and Swartz (1997) [64]

Goodson and Katre (1997) [65]



Heavy maleimide derivatives for SDS–PAGE (shift in mobility)
AMS (4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid) disodium salt

PEG-mal (polyethylene glycol maleimides): Methoxypolyethylene glycol maleimide

are the benzofurazans SBD-F (ammonium 7-fluoro 2,1,3-benzooxadiazole-4-sulfonate) and ABD-F (4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole) [68].The great advantage of derivatizing with benzofurazans instead of mBBr lies in that they emit light only when linked to thiols, whereas mBBr has a weak fluorescence of its own that gives rise to system peaks on chromatograms. In addition, with benzofurazans there is no need to remove excess reductants, while mBBr has the drawback of reacting with both phosphines and thiol-based reductants. It is worth noting that SBD-F labeling needs a higher pH and temperature (60 °C), and longer incubation times (1 h), whereas ABD-F reacts at room temperature and within 10 min at pH 8 [69]. 5-IAF (5-iodoacetamidofluorescein) is a halide that quickly reacts with thiols at room temperature at pH 12.5. It is used in the capillary electrophoretic analysis of various thiols [70]. Other fluorescent derivatives are listed in Table 1.

To analyze the different redox states of LMW thiols, an additional alkylation step is usually needed before reducing and derivatizing the oxidized thiols. Briefly, reduced thiols are irreversibly alkylated with N-ethylmaleimide (NEM) [59], 2-vinylpyridine (VP) [71], or iodoacetic acid (IAA) and iodoacetamide (IAM) [72]. Excess alkylating agent is then removed to avoid any alkylation of newly-reduced thiols, and this is usually done by phase separation with ethers (e.g. petrol or diethyl ether), or by acid precipitation. The sample containing the oxidized thiol is then reduced and derivatized again using a different labeling strategy. Then the total and oxidized thiols are measured, and the reduced fraction is obtained by subtraction. Alternatively, when studying aminothiols (such as Hcys, GSH and GSH homologs), the oxidized and reduced thiol can also be detected simultaneously, without the reducing step, by using a non-thiol reagent like OPA (ortho-phthalaldehyde), specific for primary amines. For example, when studying GSH and GSSG simultaneously, the sample can be labeled first with NEM (reacting with reduced GSH to prevent further oxidation during manipulation), and then with OPA (reacting with both GSH and GSSG [68,73].

Separation

Labeled LMW thiols are separated using two different strategies, electrophoresis (capillary and two-dimensional) [67,74] or, more often than not, chromatography. The most commonly-used technique involves high-performance liquid chromatography (HPLC) separation followed by fluorescence detection, due to its high sensitivity. Other chromatographic methods used in this setting include thiol-selective affinity chromatography (e.g. solid phase extraction, SPE) [75], and gas chromatography (GC), which is particularly indicated for detecting volatile thiols [76,77].

Detection

Depending on the derivatizing strategy adopted, UV or fluorescence detectors are coupled to the chromatographic system used for thiol separation. Electrochemical detection requires no derivatizing steps and consists in coupling the HPLC to amperometric or coulometric detectors [78]. In all these cases, the thiols are identified by comparing the peak retention times on the chromatograms obtained with the samples with standard ones. The thiols can also be quantified by analyzing the peak areas and using thiol-specific calibration curves.

HPLC and GC can also be coupled with mass spectrometers (MS) for a more accurate identification (LC–MS and GC–MS), where GC–MS is particularly indicated for analyzing volatile thiols [68,79,80]. Guan and colleagues developed a method using LC–MS for simultaneously detecting and quantifying GSH, GSSG, Cys, Hcys and their disulfides in biological samples derivatized with Ellman's

reagent [81]; other reagents can be used providing the modification is stable and produces a definite fragmentation pattern. Of course, MS analysis can also be preceded by *ad hoc* separation techniques, e.g. trap-and-release membrane introduction mass spectrometry (T&R-MIMS) [82].

Redox proteomic methods have been developed on MS instrumentation for the purpose of analyzing protein thiolation. As shown in Fig. 2, the analysis is generally performed by using two derivatizing steps: the first one protects (i.e. modifies) free, reduced Cys, while the second acts on Cys originally involved in disulfide bridges. Then protein digestion is performed before the peptides are identified using MS [83,84]. The drawbacks of this type of protocol are that Cys linked to a LMW thiol cannot be distinguished from Cys involved in structural disulfide bonds, and different thiolation PTMs cannot be distinguished from one another. Ansong et al. distinguished between glutathionylation and cysteinylation modifications on the same Cys residue in bacterial samples by skipping the derivatizing and reducing steps, and performing directly LC–MS analyses on digested samples [34].

Non-protein, LMW thiols in plants

Thiols protect cell components working as redox buffers against a variety of reactive chemical species, such as reactive oxygen and nitrogen species, metals, xenobiotics, and other reactive electrophilic species [85]. A handful of LMW thiols are known to occur in plant cells, but recent data suggest that there are hundreds of them [1]. Most of these LMW thiols derive from Cys or GSH (Fig. 1), the latter being the most abundant. Fig. 3 lists the structures and molecular masses of the thiols described in the following paragraphs; the concentrations reported in literature are listed in Table 2.

Cysteine

Cys is the main product of plant sulfur assimilation. Besides being a component of thiol-containing proteins, in which it has both structural and functional roles (see Thiol properties section), it is a core metabolite that serves as a sulfur donor for a number of compounds such as Met, vitamins (thiamine and biotin), LA, coenzyme A, GSH, and many others (Fig. 1).

Cys is synthesized in two steps: first, an acetyltransferase catalyzes the acetylation of serine from acetyl-CoA, producing O-acetylserine, then an O-acetylserine-(thiol)-lyase adds the reduced sulfur to O-acetylserine by eliminating the acetate moiety and forming Cys (Fig. 4). The first step occurs mainly in the mitochondria, the second in the cytosol and chloroplasts [9]. As already discussed, Cys concentrations are usually low because it is rapidly incorporated in proteins or converted into other compounds, especially GSH [4]. At high concentrations (above 50 μM), Cys is considered toxic. The mechanisms by which Cys can have toxic effects are an irreversible thiol oxidization that leads to a loss of sulfur, and the formation of complexes with metal ions that can trigger Fenton reactions and the formation of hydroxyl radicals [98–100].

Cys can reversibly dimerize into cystine through a disulfide bond. The metabolism of cystine in plants is still not fully understood: while both cystine transporters and reductases are known in mammals, in plants only a cystine reductase has been described in pea seeds, but its complete characterization is still lacking. It has been hypothesized that cystine can be reduced by GSH, or by enzymes such as Trxs, Grxs or GSH reductases, but several studies have reported that GSH reductases cannot reduce cystine [100–102]. In *Arabidopsis*, on the other hand, a cystine lyase reportedly catalyzes the cleavage of cystine's β-carbon-sulfide link, resulting in the release of thiocysteine, pyruvate, and ammonia. Thiocysteine is a LMW thiol compound consisting of a Cys linked to a sulfhydryl moiety through a disulfide bond, and can be further metabolized into cystine or thiocyanate, hydrogen sulfide, iron-sulfur clusters for protein assembly, or elemental sulfur [103].

The acetylation of Cys to form N-acetylcysteine (NAC) has yet to be described in plants, but this compound has been reported in several vegetables, including garlic, onion, peppers, and asparagus [104,105]. Synthetic NAC is currently used as a nutritional supplement and drug in humans for its antioxidant properties.

Finally, we should mention that although they are not thiols – there are several non-protein alkyl-Cys and alkyl-Cys-sulfoxides in plants (especially in *Amaryllidaceae, Brassicaceae*, and *Leguminosae*) that may act as precursors for the release of volatile thiols. Much of the characteristic odor associated with most of these plants is due to the degradation of Cys derivatives by specific lyases [106]. Other volatile thiol compounds derived from Cys are discussed in Diversity of LMW thiols in plants section.

Homocysteine

In plants, Hcys is an intermediate in the biosynthetic pathway of Met. Cys is the sulfur donor, which is transferred to Met via a three-step mechanism. First of all, Cys and O-phosphohomoserine (OPHS) are coupled to form the thioether cystathionine, which is rapidly converted into Hcys with the concomitant formation of pyruvate and ammonia (Fig. 4) [4,107]. The enzymes required for the first and second steps are cystathionine- γ -synthase (Cgs) and cystathionine β-lyase (CbL), respectively, which are believed to share the same ancestral origin [108,109]. These reactions occur in plastids and then Hcys is transported to the cytosol via an unknown mechanism [4]. The Met-synthase enzyme methylates Hcys to form Met by using N5-methyltetrahydrofolate as a methyl group donor. Then Met can be transported into the plastids again or remain in the cytosol, where it is involved in other pathways, such as protein synthesis or conversion to SAM, which serves as a methyl donor for a number of molecules [4,110]. The synthesis of Met (and its intermediate, Hcys) is controlled by the competition between Cgs and threonine synthase, since they need the same OPHS substrate to form cystathionine or threonine, respectively [109]. When Met is not used for protein synthesis, it can be regenerated through the SAM cycle: specific methylases use SAM as a methyl donor group to produce methylated molecules and S-adenosylhomocysteine, which is then enzymatically hydrolyzed to adenosine and Hcys [11].

There is an alternative biosynthetic pathway for the direct formation of Hcys from OPHS. Instead of Cys, the sulfur donor is the sulfide, which is added to OPHS directly by an enzyme with a sulfhydrylase activity. This pathway only takes part in 3% of all Hcys synthesis, however, and is physiologically insignificant [111–113]. In humans, Hcys has received considerable attention because high levels in plasma represent a risk factor for cardiovascular diseases such as atherosclerosis and venous thrombosis [81].

Synthesis and degradation: glutathione, gamma-glutamylcysteine and cysteinylglycine

Glutathione (GSH; γ-glutamyl-cysteine-glycine,) is a key molecule with an essential role in cellular homeostasis. Its properties and regulation have attracted the attention of scientists worldwide, as documented by the vast body of literature on the topic [114–117]. In plant cells, GSH is thought to occur at concentrations between 3 and 10 mM, and it is found in the major cellular compartments [7].

It is the main non-protein LMW thiol in plants, containing a gamma peptide linkage between the amine group of cysteine and the carboxyl group of the glutamate side-chain; as such, it cannot

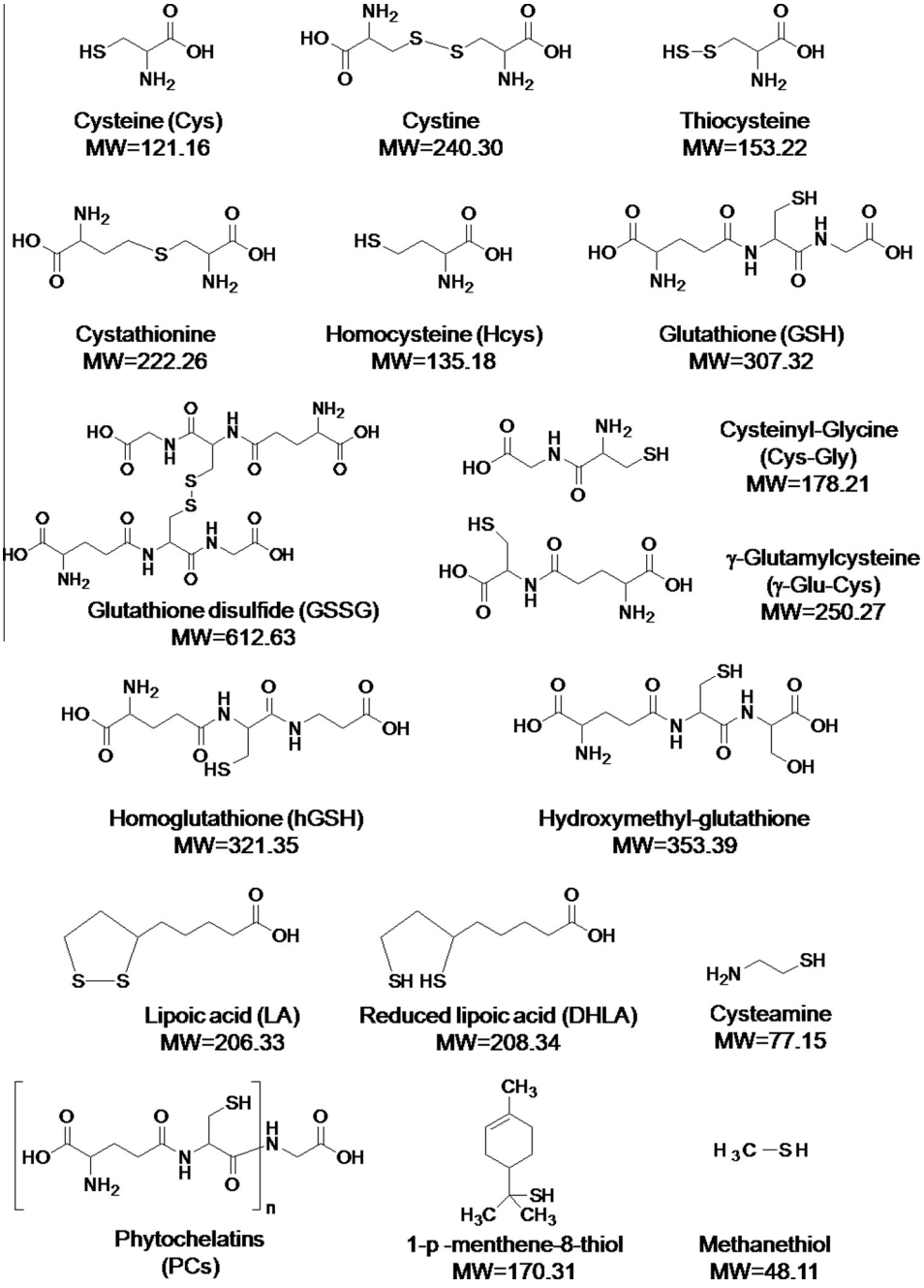


Fig. 3. The structure and molecular weight of the best-known LMW thiols in plants.

be a substrate for proteases. This molecule exposes the —SH group of the free cysteine, which can be oxidized to form a dimer (GSSG) held by a disulfide bond between two identical molecules. The ratio between GSH and GSSG is an important indicator of the cell's redox state. Under physiological conditions, the intracellular glutathione pool is kept in its reduced form, but GSSG can accumulate under conditions of oxidative stress.

GSH being the most abundant thiol controlling the redox potential of the major cellular components, the GSH redox state in turn modulates the reduction state of the thiol groups of susceptible enzymes via thiol/disulfide exchange reactions [118].

In plant physiology, GSH is involved in regulating cellular metabolism, with an important role in protecting against oxidative stress, as an antioxidant, preventing damage caused by bioreactive oxygen species. It also participates in xenobiotic and heavy metal detoxification, plant-pathogen interactions, and plant growth. As a component of sulfur metabolism, it serves as a molecule for the storage of reduced sulfur and its long-distance transport between different organs. It is a cofactor of adenosine-phosphosulfate reductase (APR) in the biosynthesis of Cys, and a precursor in the biosynthesis of PCs and glucosinolates [114], and it is needed for the maturation of iron-sulfur proteins [119]. The GSH biosynthesis pathway in plants is essentially similar to the one described in other organisms (Fig. 5) [120–122]. Two ATP-dependent enzymes (GSH1 and GSH2) produce GSH sequentially from Glu, Cys and Gly. In the first step, the intermediate γ-glutamylcysteine (γ-Glu-Cys) is synthesized in the plastid; following γ-Glu-Cys export across the chloroplast envelope, the addition of glycine can occur in both chloroplasts and cytosol. In its active form, the γ-Glu-Cys synthetase (GSH1) enzyme forms a homodimer linked by two

Table 2
Concentrations and localization of LMW thiols in plant tissues reported in literature.

LMW thiols	Localization	Concentration	References
Cys	*Zea mays* root, cell sap	56 μM	Miller, 1985 [86]
	Zea mays root, exudate	12 ± 4 μM	Miller, 1985 [86]
	Plants (general)	⩽10 uM	Leustek al., 2000 [87]
	Arabidopsis leaves, cytosol	11 ± 2 μM	Yarmolinsky et al., 2013 [88]
Hcys	Wheat (grain/seed)	390 μg/100 g	Ruseva et al., 2014 [89]
GSH	All major cellular compartments (general)	3–10 mM	Leustek and Saito, 1999 [7]
	Chloroplasts (general)	1–4.5 mM	Noctor and Foyer, 1998 [90]
	Arabidopsis root, cytoplasm from different cell types	2–3 mM	Fricker et al., 2000 [91]
	Poplar (*Populus*) leaves, cytosol of both photosynthetic and non-photosynthetic cells	0.2–0.3 mM	Hartmann et al., 2003 [92]
	Arabidopsis leaves	0.45 ± 0.01 mM	Yarmolinsky et al., 2013 [88]
	Arabidopsis leaves	855.72 ± 44.61 μM	Tolin et al., 2013 [93]
	Medicago sativa young leaves	59 ± 10 μM	Pasternak et al., 2014 [94]
	Medicago sativa mature leaves	16 ± 6 μM	Pasternak et al., 2014 [94]
Cys-Gly	Arabidopsis leaves	5.03 ± 0.14 μM	Tolin et al., 2013 [93]
	Barley root	1.1 ± 0.3 μM	Ferretti et al., 2008 [95]
	Zea mays leaves	1.2–1.6 μM	Masi et al., 2002 [96]
γ-Glu-Cys	*Zea mays* leaves (light hours)	2–3 μM	Masi et al., 2002 [96]
	Zea mays leaves (dark hours)	7–8 μM	Masi et al., 2002 [96]
hGSH	Medicago sativa young leaves	0.67 ± 0.07 mM	Pasternak et al., 2014 [94]
	Medicago sativa mature leaves	1.67 ± 0.05 mM	Pasternak et al., 2014 [94]
LA	Leaves (15-day-old wheat seedlings)	4.75 ± 0.24 mM	Sgherri et al., 2002 [97]
	Roots (15-day-old wheat seedlings)	7.22 ± 0.36 mM	Sgherri et al., 2002 [97]
DHLA	Leaves (15-day-old wheat seedlings)	13.77 ± 0.69 mM	Sgherri et al., 2002 [97]
	Roots (15-day-old wheat seedlings)	45.37 ± 2.27 mM	Sgherri et al., 2002 [97]

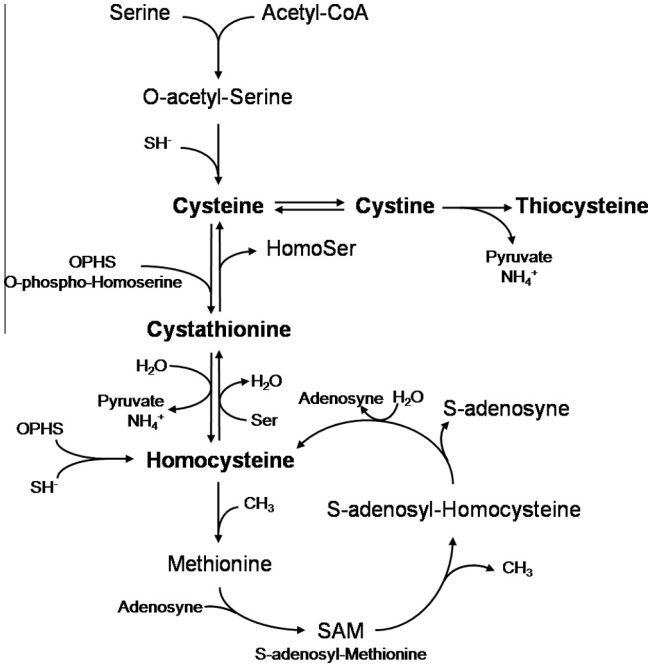


Fig. 4. Cys biosynthesis pathway, adapted from the Arabidopsis KEGG "Cysteine and Methionine Metabolism" and "Sulfur metabolism" available online http://www.genome.jp/kegg-bin/show_pathway?ath00270 and http://www.genome.jp/kegg-bin/show_pathway?ath00920.

disulfide bonds [123], which are probably implicated in the up-regulation of GSH synthesis in response to oxidative stress.

Functional genomic studies using *A. thaliana* mutants indicate that a reduced GSH content results in: failure to develop a root apical meristem; a disrupted auxin transport and metabolism; loss of apical dominance and reduced secondary root production; greater sensitivity to cadmium; a decreased camalexin content; and an enhanced sensitivity to pathogens. Oxidative stress conditions and an increased H_2O_2 availability are also known to induce glutathione accumulation in several plant species, whereas *gsh1* and *gsh2* genes respond to jasmonic acid, heavy metals and light treatments, and to conditions of stress such as drought and pathogens [114].

GSH in plant cells is degraded by GGT (γ-glutamyl transferase/transpeptidase) and GGCT (γ-glutamyl cyclo-transferase) activity. These two alternative degradation pathways are located in different compartments. GGTs are extra-cytosolic; in *Arabidopsis*, there are two known apoplastic isoforms, GGT1 and GGT2, the former located in the cell wall and the latter attached to the plasma membrane, that take part in the γ-glutamyl cycle involving GSH extrusion to the apoplastic space, degradation to its constituent amino acids, and their re-uptake by aminoacid transporters, followed by GSH resynthesis. Another isoform, GGT4, is vacuolar and assists in degrading GSH conjugates. GGTs catalyze the removal of the γ-glutamyl bond, thus releasing Glu (or γ-glutamyl-peptides) and the thiol Cys-Gly.

Rising levels of Cys-Gly in biological samples can be seen as an indicator of a greater degradation of GSH by GGT, a situation observed experimentally under conditions of stress [96]. Further confirmation of the role of GGT in response to environmental stress comes from the finding that its activity is also enhanced in *Ceratophyllum demersum* following arsenate treatment [124]. Consistently with the location of GGT, Cys-Gly is mainly extracellular; and in *Arabidopsis* mutants lacking a functional *ggt1* gene, Cys-Gly is almost abolished in the apoplast. Proteomic analyses in *ggt1* mutant leaves point to the upregulation of defense and stress response genes, giving the impression that the γ-glutamyl cycle in plants is implicated in redox sensing and redox homeostasis [93].

Some degradation of GSH to Cys-Gly may also occur spontaneously (authors' personal observations). Cys-Gly is a strong nucleophile and may thiolate proteins in animal systems (see Protein thiolation section). The same is probably true of plants, but evidence of this is lacking for the time being.

Glutathione homologs: homoglutathione and hydroxymethyl-glutathione

There have been reports of GSH analogs (Fig. 3) occurring in the plant kingdom that are not reported in any other organisms, such

as homoglutathione (hGSH, γ-Glu-Cys-β-Ala) in several members of the *Fabales* order [125], and hydroxymethyl-glutathione (γ-Glu-Cys-Ser), which is widespread in the *Poaceae* family [126]. Their functions are similar to those of GSH [127], and their occurrence in phloem sap demonstrates that they both serve as major reduced sulfur transporters in whole plants.

Recent literature points to hGSH also having a role in establishing the host–parasite/symbiont relationship. hGSH has been shown to enhance the expression of salicylic acid (SA), and to induce changes in water transport and salicylic acid signaling pathways, thus interfering with the proper development of the symbiotic interaction between *Medicago truncatula* and *Sinorhizobium meliloti* [128]. Together with GSH, hGSH has a critical role in the nodulation process [31], in nitrogen fixation in *M. truncatula* nodules [129], and in root-knot nematode development [130].

The conjugation of hGSH to the herbicide fomesan confers tolerance in soybean. The expression of a hGSH synthetase together with a hGSH-preferring GST from soybean was used to confer resistance in tobacco, which is sensitive to fomesan [131].

Cysteamine

Cysteamine (also called mercaptamine or β-mercaptoethylamine) is the simplest aminothiol and it is produced by two alternative biosynthetic pathways (Fig. 6): Cys decarboxylation or coenzyme A degradation, the latter has been described in animals and it is not clear yet whether it occurs also in plant [68]. Coenzyme A is degraded to pantetheine, the breakdown of which produces cysteamine, which is rapidly oxidized into hypotaurine by the enzyme cysteamine dioxygenase [132].

Cysteamine reportedly stimulates GSH synthesis, but its main role is in the synthesis of taurine, through the intermediate hypotaurine, which can also be produced by Cys oxidation [133,134]. Since both Cys and cysteamine are cytotoxic at high concentrations, they are rapidly converted into taurine [135]. The taurine biosynthetic pathway has been characterized in mammals, but it has also been detected in plants and prokaryotes, in which the mechanisms of synthesis have yet to be thoroughly elucidated [136].

The role of cysteamine in plants is not entirely clear, but there is *in vitro* evidence to indicate that RuBisCO, an enzyme involved in the Calvin Cycle, can be completely inactivated with cystamine/cysteamine buffers (cystamine is the dimeric, oxidized form of cysteamine). In conditions of oxidative stress, it first becomes inactive, and then it becomes sensitive to proteases as a result of several conformational changes affecting cysteines. These processes demonstrated *in vitro* suggest an *in vivo* involvement of cysteamine in oxidative stress and in senescence processes [137].

There is still a general shortage of knowledge on this compound, but cysteamine may have regulatory and physiological functions. We have found cysteamine as a major LMW thiol in stored apple skins, more abundant even than GSH (Fig. 7). It could be very important to learn more about the process of cysteamine biosynthesis and its metabolism in plants because cysteamine may have been one of the most abundant and stable thiols available on the primitive Earth as demonstrated by Miller and Schlesinger [138].

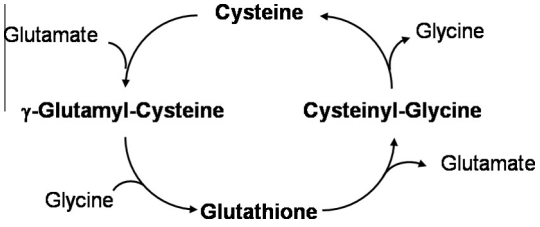


Fig. 5. GSH biosynthesis pathway adapted from the Arabidopsis KEGG "Cysteine and Methionine Metabolism" available online http://www.genome.jp/kegg-bin/show_pathway?ath00270.

Phytochelatins

PCs are small, Cys-rich polypeptides synthesized from GSH through a PC synthase (γ-glutamylcysteine dipeptidyltranspeptidase) in response to high concentrations of toxic metals in the cell's cytoplasm as follows [139,140]:

Step I: γ-Glu-Cys-Gly → γ-Glu-Cys + Gly
Step II: γ-Glu-Cys + $(\gamma\text{-Glu-Cys})_n$-Gly → $(\gamma\text{-Glu-Cys})_{n+1}$-Gly

The general structure of PCs is $(\gamma\text{-Glu-Cys})_n$-Gly, with increasing repetitions of the dipeptide Glu-Cys linked through a γ-carboxylamide bond (Fig. 3), where *n* can range from 2 to 11, but is typically no more than 5 [141,142]. Based on the repetitions of this dipeptide, PCs are classified as PC2, PC3, PC4, etc. The terminal amino acid is usually Gly, but there are variants in some plant species, such as $(\gamma\text{-Glu-Cys})_n$-β-Ala, $(\gamma\text{-Glu-Cys})_n$-Ser, and $(\gamma\text{-Glu-Cys})_n$-Glu. Irrespective of these differences, all PCs are involved in metal homeostasis and detoxification, i.e. they have the ability to transport heavy metals into the vacuole by means of specialized transporters [140]. They also play an important part in maintaining the ionic homeostasis of the cell [143].

PCs help with detoxification by forming metal-PC complexes with the Cys thiol group and thus sequestering the heavy metals in the cytosol. The heavy metals can be Cd, Cu, Hg, As and Pb, and each one induces different levels of PC expression [142]. They occur in higher plants, marine and freshwater algae, some fungi, lichens and some animal species, responding particularly to Cd [144,145].

Lipoic acid

LA, or α-LA (5-(1,2-dithiolan-3-yl)-pentanoic acid), is an amphipathic sulfur-containing molecule found in prokaryotic microorganisms, animals and plants [146]. It was first isolated by Reed and colleagues from bovine liver in 1951 [147].

Due to its structural properties (Fig. 3), LA is soluble in both water and organic solvents, with a preference for the latter. It can neutralize ROS and reduce oxidized forms of other antioxidants, and that is why it is called a super-antioxidant. LA contains a single chiral center and an asymmetric carbon, resulting in two enantiomers, R-LA and S-LA, the first of which is the essential cofactor synthesized in cells. In its reduced form, DHLA, there are two free thiol groups in each molecule and it is the predominant form serving as an antioxidant, by interacting with ROS in conditions of stress and reducing GSH [148]. It can also act as a

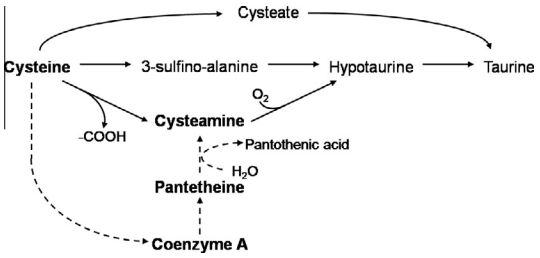


Fig. 6. Cysteamine biosynthesis pathway adapted from the Arabidopsis KEGG "Taurine and hypotaurine biosynthesis" available online http://www.genome.jp/kegg-bin/show_pathway?ath00430. Dotted arrows refer to a molecular pathway described in mammals by Coloso et al..

pro-oxidant, however, by being iron-reducing and generating S-containing radicals that can damage proteins [149,150]. The oxidized form contains the two S atoms connected by a disulfide bridge. The special position of the two sulfur atoms in the molecule gives LA a marked tendency for reduction. DHLA has vicinal thiols, thus making it more easily oxidized than monothiols, and making this molecule very active in exchange reactions [151].

It is an important cofactor for the activity of several multienzyme complexes such as pyruvate dehydrogenase (responsible of the production of acetyl-CoA), and glycine decarboxylase; complexes involved in the oxidative decarboxylation of α-ketoacids and in the glycine cleavage system [152]. In these multienzyme complexes, LA is covalently bound via an amide linkage to the ε-amino group of specific lysine residues (which are highly conserved) in the subunit E2 of the multienzymatic complex [153]. This function of LA is very important in energy metabolism as part of the complexes regulating carbon flow into the Kreb's cycle and ultimately producing ATP [154].

LA is synthesized in mitochondria in both animal and plant cells, but in plants a lipoic acid synthase has been located also in plastids [153]. LA biosynthetic pathway has yet to be thoroughly clarified in any organism, and most of collected information comes from bacteria. The direct precursor of LA is octanoic acid (from fatty acid biosynthesis) linked to an acyl carrier protein, while the sulfur donor is less certain, and presumably could be iron-sulfur cluster or SAM [155,156]. In *A. thaliana* it has been demonstrated that the *LIP1* cDNA encodes a LA synthase, with very similar sequences to those identified in *Escherichia coli* and yeast, but little is known about its mechanism. For sure, LA is first synthesized as linked to an acyl carrier protein and then released/transferred to a target protein [152,157].

Plant and animal tissues contain small amounts of LA. The most abundant plant sources of LA are spinach, broccoli, tomatoes, brussels sprouts, potatoes, garden peas and rice bran [149]. All the properties of LA make it a very useful agent in the treatment of many diseases, including diabetes, atherosclerosis, degenerative processes in neurons, cataract formation, radiation injury, cancer, and acquired immune deficiency syndrome (AIDS). It is also used in anti-age treatments [158].

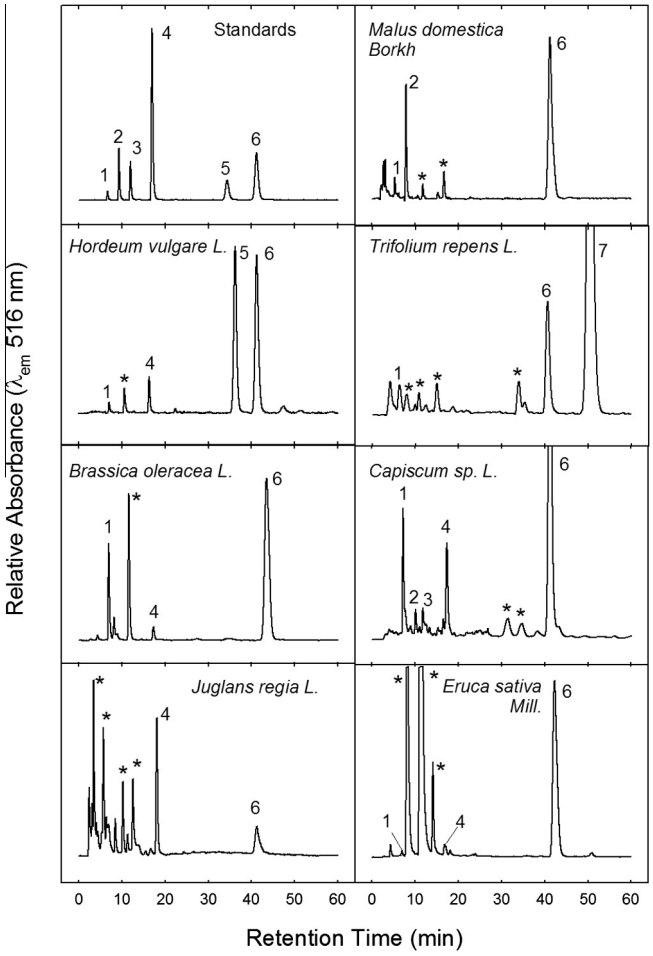


Fig. 7. Representative chromatographic separation of LMW thiols from several plant samples, after derivatization with SBD-F: Standards, *Malus domestica Borkh* skin, *Hordeum vulgare* L. root, *Trifolium repens* L., *Brassica oleracea* L., *Capiscum* sp. L., *Juglans regia* L., *Eruca sativa* Mill. Numbers refer to: 1 Cys; 2 Cysteamine; 3 Hcys; 4 Cys-Gly; 5 γ -Glu-Cys; 6 GSH; 7 putative hGSH, * unknown LMW thiols.

Volatile thiols

Significant amounts of volatile thiols are produced by secondary metabolism in specific plant species and they have an important role as food flavorings. However, most of the sulfur flavoring agents originate not directly from plant biosynthetic pathways, but from fermentation processes or further preparation procedures. To give an idea of the variety of LMW sulfur compounds contained in foods, be they synthetic or derived from natural sources, at least 188 have been classified and tested by the European Food Safety Authority [159], at least fifty of which are thiol molecules, classified as: (i) simple thiols with un-oxidized aliphatic or aromatic side-chains; (ii) thiols with oxidized side chains, in which an alcohol, aldehyde, ketone, ester, or carboxylic acid group is present; and (iii) dithiols [159].

The most often studied volatile thiols are those contained in fermented beverages, and especially wine. They are classified as varietal aroma compounds, i.e. molecules synthesized by the plant and occurring in grape fruits in free form or linked to a non-volatile molecule (the cleavage occurs during wine production but the original moiety produced by the plant is preserved), and pre-, post- or fermentation aromas. Odoriferous varietal aromas released by the fermentation process often have a cysteinylated or a glutathionylated odorless precursor synthesized by the grape berry [160]. In particular, cysteinylated precursors are plentiful in plants, providing an abundant source of aroma for the food industry [161]. Other compounds do not have a Cys or GSH-precursor, for example 1-p-menthene-8-thiol (Fig. 3) occurs in the intact plant (*Vitis vinifera*), and derives from the reaction of limonene with SH_2 [162].

There are also many unpleasant odors associated with volatile thiols, generally deriving from the degradation of Cys, Met or other larger sulfur-containing molecules. Methanethiol (Fig. 3) is plentiful in cabbage and other *Brassicaceae* [163], where it is produced from bisulfide by specific methyltransferases, but it is also found in other plant species at lower concentrations (e.g. *Arabidopsis*), produced from Met by a γ-methionine-lyase [164]. Some volatile alkane thiols have also been characterized in onion (e.g. 3-mercapto-2-methylpentanal and 3-mercapto-2-methylpentan-1-ol).

The case of glucosinolates and thioglucose

Glucosinolates are a class of sulfur-containing secondary metabolites with a major role in plant defense in the *Brassicaceae* family [165], and some of them have proven anti-cancer properties in medical treatments [166]. They contain one sulfur atom derived from phosphoadenosine phosphosulfate, and another one is obtained from the amino acid Cys, or two if the starting amino acid is Met [167]. It has now been demonstrated that GSH is a precursor in the synthesis of glucosinolates and also of camalexins, but whether a cytosolic or an extracellular γ-glutamyl-peptidase, or both, are involved is still debated [168–171]. These compounds are substrates for thioglucosidases (myrosinase) but the reaction

is prevented because they are restricted to different compartments. On chewing by herbivores, this compartmentalization is lost and hydrolysis occurs, causing a cascade of reactions through thioglucose, and leading to the release of toxic compounds (e.g. thiocyanates, isothiocyanates, indoles) that defend against predators and pathogens. Thioglucose can thus be considered another intermediate thiol in plant metabolism, implicated in plant defense.

Non-enzymatic glucosinolate hydrolysis occurs at alkaline pH also under the analytical conditions imposed for thiol reduction (see LMW thiol separation and detection techniques section), which results in the spontaneous release of thioglucose [172]. This molecule is therefore valuable as a means for rapidly estimating total glucosinolate content [173].

Diversity of LMW thiols in plants

Gläser and colleagues ascertained that there were about 300 sulfur metabolites in *Arabidopsis* using MS techniques; most of them remain unidentified, and many of these could be LMW thiols [1]. Indeed, chromatographic separations performed at our lab revealed the existence of several unknown molecules containing thiols in several plant species, including fruit and vegetables. Fig. 7 shows some representative HPLC chromatograms of a series of vegetable and other plant samples after derivatization with SBD-F, some of which show unknown thiols that are specific to some plants, while others exhibit organ/tissue specificity.

Conclusions and future prospects

LMW thiol molecules are biologically relevant due to the intrinsic reactivity of the nucleophilic -SH moiety. By participating in reversible redox reactions, they can modify the redox state of sensitive molecules and the cellular environment. They can conjugate or make complexes with xenobiotics and toxic compounds, and they can deactivate them. They can post-translationally modify regulatory enzymes and control metabolism. They may also be technologically relevant, with implications for food quality and safety, and a possible fallout on human health.

Despite the numerous implications relating to our understanding of LMW thiol metabolism, it is no exaggeration to say that many of them have been neglected so far, and a great deal of work remains to be done in this field. We can outline at least three areas that deserve further investigation.

(i) The identification of new compounds. While a few LMW thiols have been described in the literature, a huge and diverse array of unknown thiol molecules clearly exist in plant biology, as evidenced chromatographically by thiol-specific derivatives with fluorescent dyes (Fig. 7). Identifying these thiols represents a major challenge, given that in most cases they are hardly abundant – probably in the range of micromolar or sub-micromolar concentrations. A new, upcoming generation of mass spectrometers with a high sensitivity and resolution, combined with the development of thiol purification and concentration protocols, will be of great help in the process of their identification.

(ii) The identification of protein residues modified by glutathionylation. It has been demonstrated that glutathionylation regulates a significant number of biological processes, including photosynthesis, germination, seed development and desiccation, but few studies have focused on experimentally verified protein glutathionylation sites. Implementing this knowledge by means of proteomic studies is a necessary step in order to make progress in our understanding of the biological meaning of such modifications, to identify the part played by GSH when linked to the proteins, and how these modifications are regulated.

(iii) The investigation of protein thiolation. Glutathionylation is the most widespread and significant thiolation modification, but it has recently been demonstrated in both mammals and bacteria that other LMW thiols can be bound to proteins, such as Cys-Gly and Cys. In particular, such modifications may occur on the same residues where glutathionylation takes place, modulating different processes depending on which LMW thiol is linked. In the light of these findings, it would be well worth studying disulfide PTMs. MS could be the most appropriate technique for this purpose at present, but new methods need to be developed because standard protocols generally have to include a reducing step.

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