Summary

Sulfur is an essential element for plant primary metabolism as a structural component of proteins and lipids, antioxidants, regulatory molecules, metal-binding molecules and cofactors/coenzymes. The various steps involved in the reduction of sulfate and its assimilation into cysteine happen predominantly or exclusively in plastids. Cysteine holds a central position in S metabolism and is used for the biosynthesis of a variety of other reduced S compounds including methionine, S-adenosylmethionine, glutathione and phytochelatins, the coenzymes thiamine, biotin, lipoic acid and Coenzyme-A, the Molybdenum cofactor and Fe-S clusters. In this chapter we will give an overview of S metabolism in higher plants, focusing on the role of plastids. The regulation of S metabolism is discussed, as well as the involvement of S metabolic pathways in metabolism of other oxyanions. We conclude with an overview of results from genetic engineering of S pathway enzymes.

I. Introduction

Sulfur (S) is an essential macronutrient for plants and is present at 0.1 to 1% of plant dry weight depending on the plant family and soil type (Marschner, 1995). Sulfur is generally less limiting for plant growth than other macronutrients such as N or P, but nevertheless, positive responses to S fertilization have been reported from many areas in the world including most agricultural areas (Hoeft and Walsch, 1975). Sulfur deficiency manifests itself as chlorosis of younger leaves and stunted growth (Marschner, 1995).

The main form of S taken up by plants is sulfate, which is the most oxidized form of S (valence state +6), and the predominant bio-available form in oxic soils. The form of S present in biomolecules is mostly reduced S, although S also occurs in its oxidized form in sulfolipids and various sulfated compounds (for a review, see Leustek et al., 2000). The reduction of...
sulfate needed for the synthesis of many S compounds happens exclusively in plastids and predominantly in the photosynthetic tissues. Cysteine (Cys) is the first organic form of S after sulfate reduction. Cys holds a central position in S metabolism and is used for biosynthesis of a variety of other reduced S compounds including methionine (Met), S-adenosylmethionine (SAM), glutathione (GSH) and phytochelatins (PCs), the coenzymes thiamine, biotin, and Coenzyme-A, the Molybdenum cofactor (MoCo) and Fe-S clusters.

Sulfur compounds hold essential functions in plant primary metabolism as structural components of proteins and lipids, as antioxidants, as regulatory molecules, metal-binding molecules, and as cofactors/coenzymes for biochemical reactions. Next to this structural role, the S atom is often directly required for the function of S-containing molecules. For instance, a thiol (-SH) group in proteins or peptides may have a key function due to its redox capacity and its metal-binding properties, or be directly involved in enzyme catalysis.

In addition to the roles of S compounds in primary plant metabolism of all plants, certain S compounds are not essential but play a role in stress resistance or in ecological interactions between plants and other organisms. For instance, some S compounds are signaling molecules in plant-microbe interactions (Lerouge et al., 1990; Denarie and Cullimore, 1993). Other S compounds (e.g. glucosinolates) may also protect plants from herbivory or microbial infection (Ernst, 1990; Sendl, 1995; Lacomme and Roby, 1996). The sulfated compound gallate glucoside controls seismosnasty, which may also function in deterring herbivory (Varin et al., 1997). Accumulation of S compounds (e.g. sulfate or dimethylsulfoniumpropionate, DMSP) may also protect plants from osmotic stresses (Ernst, 1990).

The study of plant S metabolism has relevance for human society because S is a macronutrient and can be limiting for agricultural productivity. Also S compounds may contribute to plant biotic and abiotic stress resistance, positively affecting crop yields. The S content and allocation by crop plants contribute to animal and human food quality. Sulfur compounds can influence the quality of plant products by means of their flavors and odors (Thompson et al., 1986), by influencing the quality of flour (Byers et al., 1987), or by their medicinal activity (Hell, 1997). In addition, there are volatile S compounds emitted by plants that can influence climate (Kelly et al., 1994).

In this chapter we will give an overview of S metabolism in higher plants focusing on the role of plastids, the main site of S reduction. The regulation of S metabolism is discussed, as well as the involvement of S metabolic pathways in metabolism of other oxyanions. We conclude with an overview of results from genetic engineering of S pathway enzymes.

## II. Sulfur Compounds and Their Properties

The role of S in molecules is very diverse, because S can exist in multiple oxidation states (+6, +4, 0, −2), each with different chemical properties (Beinert, 2000). In this section we will give an overview of the major S-containing groups and their properties in biomolecules. We include a list of (classes of) S-containing biomolecules and their functions in Table 1. In the next section we will describe how these various S compounds are synthesized, and describe their individual roles in more detail. For an overview of S compounds in plants see also Buchanan et al. (2000, pp 826–830).

Thiols contain a –SH group. Examples are Cys and Cys-containing peptides (e.g. GSH) and proteins, as well as lipoic acid and Coenzyme-A. Thiol groups are redox active. Two –SH groups can form one S-S disulfide bond, giving up two electrons and two protons. This reversible reaction is used in cells to stabilize protein structure (especially secreted proteins) and to regulate enzyme activity (especially intracellular enzymes). The redox activity of thiol groups is also used by S compounds such as GSH to keep cell components in a reduced state and scavenge free radicals (Kunert and Foyer, 1993). The thiol group can also be

**Abbreviations:** ATP – adenosine triphosphate; APS – adenosine phosphoselenenate; CbS – cystathionine-β-lyase; Cgs – cystathionine-γ-synthase; Cys – cysteine; CysD – cysteine desulfurase; DMS – dimethyl sulfide; DMSe – dimethylselenide; DMSP – dimethyl sulfoniumpropionate; DMSeP – dimethyl selenoniumpropionate; ECS – γ-glutamylcysteine synthetase; ER – endoplasmic reticulum; GR – glutathione reductase; GS – glutathione synthetase; GSH – glutathione (reduced); GSSG – glutathione (oxidized); Met – methionine; MMT – methionine methyltransferase; MoCo – molybdenum cofactor; NADPH – nicotinamide adenine dinucleotide phosphate; OAS – O-acetylserine; OPH – O-phosphohomoserine; PAPS – phosphoadenosine phosphosulfate; PC – phytochelatin; PS – phytochelatin synthase; RTPCR – reverse transcription polymerase chain reaction; SAM – S-adenosylmethionine (also called AdoMet); SAT – serine acetyltransferase; ScCys – selenocysteine; SMT – selenocysteine methyltransferase; TS – threonine synthase; SQDG – sulfolipid; 6-sulfo-α-D-glucosyl diacylglycerol.

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Table 1. Overview of the main S-containing biomolecules in plants and their functions

<table>
<thead>
<tr>
<th>Molecule</th>
<th>S group</th>
<th>Function</th>
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<tbody>
<tr>
<td>Cysteine</td>
<td>thiol</td>
<td>amino acid, metabolic intermediate</td>
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<tr>
<td>Cysteine derivatives</td>
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<tr>
<td>methyl-cysteine</td>
<td>thiol</td>
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<tr>
<td>cystathionine</td>
<td>thioether</td>
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<tr>
<td>cystine</td>
<td>disulfide</td>
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<tr>
<td>glutathione</td>
<td>thiol</td>
<td>reduction processes, stress resistance</td>
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<tr>
<td>phytochelatins</td>
<td>thiol, disulfides</td>
<td>metal tolerance</td>
</tr>
<tr>
<td>Cys in proteins</td>
<td>thioether</td>
<td>cofactor of enzymes</td>
</tr>
<tr>
<td>Mo cofactor</td>
<td>thioether</td>
<td>carbon dioxide transfer</td>
</tr>
<tr>
<td>Biotin</td>
<td>thioether</td>
<td></td>
</tr>
<tr>
<td>Thiamine</td>
<td>thioether</td>
<td>coenzyme A formation?</td>
</tr>
<tr>
<td>FeS clusters</td>
<td>sulfide</td>
<td>cofactor of enzymes</td>
</tr>
<tr>
<td>Lipoic acid</td>
<td>thiol</td>
<td>coenzyme</td>
</tr>
<tr>
<td>Coenzyme A</td>
<td>thiol</td>
<td>amino acid, metabolic intermediate</td>
</tr>
<tr>
<td>Methionine</td>
<td>thioether</td>
<td></td>
</tr>
<tr>
<td>Methionine derivatives</td>
<td>methylsulfonium</td>
<td>transport, metabolic intermediate</td>
</tr>
<tr>
<td>S-adenosylmethionine</td>
<td>methylsulfonium</td>
<td>methyl donor, ethylene precursor</td>
</tr>
<tr>
<td>dimethylsulfide</td>
<td>thioether</td>
<td>release of excess S?, ecological function?</td>
</tr>
<tr>
<td>Met in proteins</td>
<td>thioether</td>
<td></td>
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<tr>
<td>Sulfolipids</td>
<td>sulfonic acid</td>
<td>thylakoid membrane component</td>
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<td>Sulfated compounds</td>
<td>sulfite ester</td>
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<tr>
<td>flavonoids</td>
<td>sulfite ester</td>
<td>drought resistance?</td>
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<tr>
<td>brassinosteroids</td>
<td>sulfite ester</td>
<td>plant growth regulator</td>
</tr>
<tr>
<td>gallate glucoside</td>
<td>sulfite ester</td>
<td>plant growth regulator</td>
</tr>
<tr>
<td>glucosinolates</td>
<td>sulfite ester</td>
<td>ecological role in defense?</td>
</tr>
<tr>
<td>peptides</td>
<td>sulfite ester</td>
<td></td>
</tr>
<tr>
<td>choline sulfate</td>
<td>sulfite ester</td>
<td>S transport? salt resistance?</td>
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</tbody>
</table>

modified during group transfer reactions such as those that involve Coenzyme-A. Furthermore, thiol groups can have a direct catalytical role in the active site of enzymes. The thiol group of Cys also has metal-binding properties and is responsible for the metal-binding capacity of many metal-binding proteins such as metallothioneins (Zhou and Goldsbrough, 1994) and metal transporters such as P-type ATPases of which there are eight in Arabidopsis (Axelsen and Palmgren, 2001). Specialized thiol compounds such as phytochelatins (PCs) are used by cells to detoxify excesses of various metals (Cobbett, 2000).

Iron-sulfur clusters contain S as sulfide (S²⁻) (Beinert et al., 1997). The most abundant forms in plants are 2Fe-2S or 4Fe-4S complexes. These cofactors are bound to thiol groups of proteins. Iron-sulfur clusters are redox active due to the capacity of Fe to reversibly take up an electron. This is used, for instance, in components of the chloroplast electron transport chain (Raven et al., 1999). FeS clusters can also have a catalytic role in enzymes other than redox activity, as occurs in aconitase.

Other S groups are found in methylsulfonium compounds, which contain S as (CH₃)₂S⁺⁻. Examples are S-adenosylmethionine (SAM, also called AdoMet), DMSP and S-methylmethionine. Furthermore, S occurs in its most oxidized state as sulfate esters (−SO₄⁻) in a variety of sulfated compounds including sulfated hormones, glucosinolates, peptides and flavonoids. Sulfur can also be present as a thioether (−S−), as in methionine, thiamine and biotin. Sulfonic acids, which contain S as −SO₃⁻, are found in glucose-6-sulfonate, cystic acid, and sulfolipids. Sulfoxides, as −SO⁻, occur in compounds such as the secondary plant compound allicin.

III. Biosynthesis and Functions of S Compounds

The flow of S in plants can be summarized as follows. Most S is taken up as sulfate, which is first activated and then reduced to sulfite and finally sulfide. Sulfide is subsequently incorporated into cysteine (Cys). Two pathways branch from this sulfite reduction pathway. Activated sulfate can be used to produce a range of sulfated compounds, and sulfite is used for the production of sulfolipids. Cys holds a central position, from which
S can go in several directions: (i) S can be transferred to methionine and its derivatives; (ii) S from Cys can be released and incorporated into various cofactors or coenzymes, and (iii) Cys can be incorporated into proteins or into the peptide glutathione and its derivatives. Cys can also be stored in methylated form. The various pathways of S metabolism, and the important role of plastids in these processes are discussed in more detail below. They are also depicted in Fig. 1 and 2.

A. From Sulfate to Cysteine

The main form of S taken up into plants by roots is sulfate. Sulfate is transported over membranes via secondary active transport by sulfate-H\(^+\) cotransporters, driven by the proton motive force created by ATPase (Smith et al., 1995). Fourteen genes encoding sulfate transporters (sulfate permeases) have been reported for Arabidopsis thaliana, which can be divided over five groups. These genes differ in tissue-specific expression.
and subcellular localization of the gene product (for a review see Hawkesford, 2003).

The assimilation of sulfate into Cys takes place mainly in the chloroplast. In C4 plants this happens in the chloroplasts of the bundle sheath cells (Schmutz and Brunold, 1984). On its way from the soil to the chloroplast, sulfate enters the plant via group-1 high-affinity sulfate transporters in the plasma membrane (Smith et al., 1995; Shibagaki et al., 2002; Yoshimoto et al., 2002). Translocation of sulfate to the shoot via the xylem appears to be facilitated by sulfate transporters from groups-4, -3 and -2 in Arabidopsis roots, involved in vacuolar efflux (sultr4;1 and 4.2) and xylem loading (sultr3;5 and 2;1) respectively (Takahashi et al., 1997, 2000; H. Takahashi, personal communication).

Sulfate is taken up from the xylem into leaf mesophyll cells, perhaps by the combined action of group 2 and 3 sulfate transporters (Takahashi et al., 1999; Grossman and Takahashi, 2001). In the cytosol the sulfate concentration is in the mM range (Schroppel-Meier and Takahashi, 2001). In the cytosol the sulfate transporters (Takahashi et al., 1997, 2000; H. Takahashi, personal communication).

Sulfate is activated by reaction with ATP to form adenosine-5′-phosphosulfate (APS). This reaction is catalyzed by ATP sulfurylase. The predominant isoform of this enzyme is located in the plastids, but there is also a minor cytosolic form. The two isoforms are regulated differently (Rotte and Leustek, 2000). In the cytosol the resulting APS is further phosphorylated by APS kinase to PAPS, which is used as a sulfate donor by cytosolic sulfotransferases. These sulfation reactions produce a variety of sulfated compounds including hormones (e.g. brassinosteroids, gallate glucosides), glucosinolates (Poulton and Moller, 1993), peptides and flavonoids (Ananvaranich et al., 1994; Lacomme and Roby, 1996; Hell, 1997).

In the chloroplasts APS is reduced by APS reductase (also called APS sulfotransferase) to sulfite (Setya et al., 1996). The two electrons probably come from glutathione (GSH) (Prior et al., 1999). Sulfite may either be used for the production of sulfolipids or further reduced to sulfide, both in the chloroplast. When used for sulfolipid production, sulfite is first coupled to UDP-glucose to form UDP-sulfoquinovose, catalyzed by the SQD1 enzyme (Sanda et al., 2001). UDP-sulfoquinovose is then coupled to diacylglycerol to form the sulfolipid SQDG (sulfolipid 6-sulfo-α-D-glucosyl diacylglycerol), which is an important component of chloroplast membranes and may contain as much as one third of leaf S (Harwood and Nicholls, 1979; Schmidt, 1986). These sulfolipids are unique to plastids and required for plastid functions including photosynthesis (Yu and Benning, 2003).

The further reduction of sulfite to sulfide is mediated by sulfite reductase, a plastidic enzyme (Bork et al., 1998). The six electrons needed for this step are thought to come from ferredoxin (Yonekura-Sakakibara et al., 2000). Sulfide is incorporated into Cys by coupling to O-acetylserylamine (OAS). This reaction is mediated by the enzyme OAS thiol lyase, also called cysteine synthase. The OAS needed for this reaction is produced by serine acetyltransferase (SAT). As only plastidic forms of APS reductase and sulfite reductase have been found, reduction of sulfate to sulfide is thought to occur exclusively in plastids. Because of the higher reducing power in the photosynthetic chloroplasts, most of sulfate reduction probably happens in chloroplasts, although non-green plastids also perform sulfate reduction. Cys synthesis from sulfide and OAS occurs predominantly in the chloroplast, although there are also cytosolic and mitochondrial isoforms of serine acetyltransferase and OAS thiol lyase (Noji et al., 1998). After formation, Cys is rapidly converted to other compounds in the chloroplast or other compartments. Therefore, the Cys concentration in the cell is quite low (μM range).

B. Cysteine in Proteins

Much of Cys is incorporated into proteins, either in the plastids or in the cytosol. Cys is coupled to a specific tRNA by an amino acyl-tRNA synthetase, delivered to a growing polypeptide chain and incorporated. Cys residues in proteins often serve an important role in protein structure and function. The structural importance is due to the capacity of two Cys thiol groups to form a disulfide bond, which can contribute to protein tertiary and quaternary structure. Disulfide bonds are formed mainly in the ER and are especially important for the structural integrity of secreted proteins, because these proteins occur in more oxidized environments (Braakman et al., 1992). In intracellular proteins, thiol groups are mostly in a reduced state, where conditions are more reducing. The reducing power of these Cys thiol groups can be used to reduce other cell components.
For instance, in chloroplasts the redox capability of Cys in thioredoxin is crucial for the regulation of photosynthetic enzymes (Buchanan et al., 2002). In the light, ferredoxin is reduced by electrons from photosystem I and in turn reduces thioredoxin, which activates stromal Calvin-Benson cycle enzymes via reduction of thiol groups. In the dark the regulatory thiol groups on these enzymes become oxidized for lack of reduced ferredoxin and thioredoxin, rendering the enzymes inactive. In this way the light reactions and the Calvin-Benson cycle of photosynthesis are synchronized to be active only in the light.

C. From Cysteine to Methionine and its Derivatives

The S in Cys can be transferred to methionine (Met) in three steps. First cystathionine-γ-synthase (CgS) couples Cys to O-phosphohomoserine (OPH) to form cystathionine. The enzyme cystathionine-β-lyase (CbL) then splits cystathionine into homocysteine, ammonia and pyruvate. These first steps occur in plastids. In the third step homocysteine is methylated by Met synthase to form Met. Met synthase has so far only been found in the cytosol. If the enzyme is also in plastids and Met has to be transported back into the plastid via as yet unknown transporters (Ravanel et al., 1998a).

Met can be incorporated into proteins either in plastids or in the cytosol. Most of the synthesized Met is converted to SAM via reaction with ATP by SAM synthetase. This may occur in the chloroplast or cytosol, but probably happens in the cytosol. SAM is then transported into plastids via an unknown transporter. SAM is involved in many reactions in different cell compartments including the plastids. It serves as methyl donor for the synthesis of a variety of plant compounds and is the precursor for the plant growth regulators ethylene and polyamines (Ravanel et al., 1998a). SAM can be reconverted to homocysteine in the cytosol. Antisense plants for one of the enzymes involved, Sadenosyl-L-homocysteine hydrolase (SAHH), showed aberrant growth and flower morphology and reduced DNA methylation (Tanaka et al., 1997) suggesting that this enzyme regulates the expression of genes involved in plant growth and flower development.

Met can be methylated in the cytosol to S-methyl-Met by the enzyme Met methyl transferase (MMT), using SAM as a methyl donor. Methyl-Met can be transported long-distance via the phloem where it can reach concentrations that are higher than GSH (Bourgis et al., 1999). Other possible fates of methyl-Met are reconversion to Met or further conversion to dimethylsulfoniopropionate (DMSP) (James et al., 1995). This compound can serve as an osmoprotectant in Spartina alterniflora (Kocsis et al., 1998). It also serves as chemosensory substrate for production of dimethylsulfide (DMS) by DMSP lyase (Dacey et al., 1987). Sulfur volatilization is a common property of plants, and may serve as a valve to get rid of excess S. Other such valves may be excretion in glands or in the rhizosphere. An alternative function for the emission or excretion of S compounds may be that they serve an allelopathic or antimicrobial function, or perhaps as signaling compounds.

D. From Cysteine to Glutathione and its Derivatives

About 2% of the organic reduced S in the plant is present in the form of non-protein thiols, and around 90% of this fraction is glutathione (γ-Glu-Cys-Gly, GSH) (Rennenberg, 1982; de Kok and Stulen, 1993). Glutathione is synthesized enzymatically in both the plastids and the cytosol (Noctor et al., 1998a). Glu and Cys are combined by γ-glutamylcysteine synthetase (γ-ECS), followed by addition of Gly by glutathione synthetase (GS). Both enzymes require ATP. Cellular GSH levels in plants can be in the millimolar range, with more than 50% localized in the chloroplasts (Foyer and Halliwell 1976; Rennenberg and Lamoureux, 1990). Two GSH molecules can be oxidized reversibly to one molecule of GSSG, yielding two protons and two electrons, which may be used to reduce other cell components. GSSG can be reduced back to GSH by glutathione reductase (GR), using NADPH as a source of protons and electrons. An isoform of glutathione reductase occurs in the chloroplast and another in the cytosol, but the predominant form is plastidic (Foyer and Halliwell, 1976). Most of glutathione is present in the reduced form in unstressed cells (Kunert and Foyer, 1993).

As described above, the thiol group of Cys enables it to reduce other molecules and to bind metals. These properties enable GSH to play many important roles in the cell (for reviews see Kunert and Foyer, 1993; Noctor et al., 1998b). Next to maintaining a reducing environment, GSH functions include storage and transport of reduced S, protection of cells against oxidative stress, detoxification of xenobiotics and heavy metals (Cobbett et al., 1998), and redox regulation of gene expression.

Glutathione may be used in the cytosol as a substrate for synthesis of phytochelatins (Grill et al., 1989).
Chapter 19 Sulfur Metabolism

Phytochelatins (PCs) are metal-binding peptides with the general structure (Glu-Cys)_n Gly, where typically n = 2 to 5 (Cobbett, 2000). They are synthesized by phytochelatin synthase, which catalyzes the elongation of the (γ-Glu-Cys)_n by transferring a γ-GluCys group from GSH to PC (or to another GSH). Phytochelatin synthase is constitutively expressed but needs to be activated by metals. Hence, PCs are only produced in response to metal stress and probably mainly function in metal tolerance, especially to Cd and As (Cobbett, 2000; Pickering et al., 2000). Following binding of metals by PCs, the complex is transported to the vacuole by an ABC-type transporter and further complexed by sulfide. The sulfide is produced in the cytosol from cysteine sulfinate and imported into the vacuole (Cobbett, 2000). Phytochelatins were shown recently to be transported from root to shoot in plants (Gong et al., 2003). The mode of PC transport over the cell membrane is yet to be elucidated. Recently a rice GSH transporter with a secondary plant compounds like anthocyanins to xenobiotics such as the herbicide atrazine (for a review about GSH transferases, see Marrs, 1996). GS-conjugates can be transported to the vacuole by the GS conjugate vacuolar pump, an ABC transporter (Rea, 1999), where they may be metabolized to Cys-conjugates (Lamoureux and Rusness, 1993).

Another fate of GSH in the cytosol is to serve as a substrate for GSH-conjugation by GSH-S-transferases (GSTs, Marrs, 1996). The GSH-S-transferases occur as a family with hundreds of members, each with different substrate specificity. Their substrates vary from secondary plant compounds like anthocyanins to xenobiotics such as the herbicide atrazine (for a review about GSH transferases, see Marrs, 1996). GS-conjugates can be transported to the vacuole by the GS conjugate vacuolar pump, an ABC transporter (Rea, 1999), where they may be metabolized to Cys-conjugates (Lamoureux and Rusness, 1993).

GSH can be transported in the phloem (Blake-Kalff et al., 1998). As mentioned above, a GSH transporter has been reported that may be involved in the transport of GSH and its derivatives over the cell membrane (Zhang et al., 2004). Glutathione can be broken down on the outer surface of the plasma membrane (and perhaps other locations) by γ-glutamyl transpeptidase, releasing Cys-Gly, which is subsequently broken down into Cys and Gly by a dipeptidase (Storozhenko et al., 2002).

E. Cysteine as a S Donor for Cofactors and Coenzymes

Cysteine can be converted to Ala and sulfide by Cys desulfurases (CysD). These are NiFIS-like proteins, which are related in structure to the NiFIS protein from Azotobacter vinelandii (Zheng et al., 1993). In Arabidopsis, one NiFIS-like enzyme has been reported in plastids (Leon et al., 2002; Pilon-Smits et al., 2002), while a second form may be present in mitochondria (Kushnir et al., 2001). Cys desulfurase enzymes function to provide reduced S for the production of Fe-S clusters as well as several coenzymes (Mihara and Esaki, 2002). In chloroplasts, Fe-S clusters play a key role in photosynthesis as well as reduction reactions. The capacity of the Fe atom in Fe-S clusters to reversibly take up an electron provides the electron carrier capacity of many components of the electron transport chain in the thylakoid membrane. Fe-S clusters are required for the function of the cytochrome b_{6}f complex (one 2Fe-2S cluster in the Rieske FeS protein), photosystem I (three 4Fe-4S clusters) and ferredoxin (one 2Fe-2S cluster) (Raven et al., 1999). The capacity of the Fe atom in Fe-S clusters to be reversibly reduced and oxidized is also used in chloroplast reduction pathways such as nitrite reductase and sulfite reductase (Lancaster et al., 1979; Krueger and Siegel, 1982).

Assembly of the FeS cluster into freshly imported ferredoxin precursor, obtained by in vitro translation, was demonstrated using isolated intact chloroplasts (Li et al., 1990). The reaction proceeds in the absence of cytosol (Pilon et al., 1995). These experiments strongly suggest the presence of an Fe-S cluster formation machinery in chloroplasts. Since Cys was identified as a source for Fe-S formation in chloroplasts (Takahashi et al., 1986; Takahashi et al., 1990), a protein with Cys desulfurase activity is likely involved in this process. Indeed, the plastidic Cys desulfurase from A. thaliana shows capacity for in vitro reconstitution of apo-Fd to Fd, and thus may serve a role in providing sulfide for Fe-S cluster formation (H. Ye, M. Pilon and E.A.H. Pilon-Smits, unpublished results).

Cys desulfurase may also provide reduced S for synthesis of the coenzymes biotin, thiamine, Molybdenum cofactor (MoCo), lipoic acid and Coenzyme-A, by analogy with bacterial pathways (Amrani et al., 2000; Leimkuhler and Rajagopalan, 2001; Mihara et al., 2002). In plants, MoCo is an essential component of nitrate reductase and several other enzymes. MoCo deficiency leads to reduced nitrate reductase activity and N depletion, as well as reduced phytohormone synthesis (Stallmeyer et al., 1999). Cys provides S for production of the pantetheine moiety of Coenzyme-A, which has many important functions in primary plant metabolism (Kupke et al., 2003). Thiamine and lipoic acid are involved in the decarboxylation of pyruvate to acetyl-Coenzyme-A. Biotin is the carrier for activated CO}_{2} in the conversion of acetyl-CoA to malonyl-CoA in fatty
acid synthesis (Wood and Barden, 1977). The thiamine biosynthesis pathway is thought to be located in the chloroplast (Belanger et al., 1995).

F. Other Fates of S in Cysteine

Two Cys molecules can be reduced reversibly via a disulfide bridge to form cystine, which may function in keeping other cell components reduced or play a role in storage of S. Another fate for Cys is methylation (or alkylation) to S-methyl-Cys (alkyl-Cys). This reaction probably occurs in the cytosol by Cys methyltransferase (Thompson and Gering, 1966). The function could be S storage, because this is a non-protein amino acid.

There are a variety of additional secondary S compounds that may be derived from Cys and that occur only in certain species. These compounds may play an ecological role by warding off microbial infection or herbivory (Virtanen, 1965; Ernst, 1990; Sendl, 1995). Relatively little is known about the biosynthesis of many of these compounds, some of which have important applications as pharmaceuticals (Hell, 1997).

IV. Regulation of S Metabolism

A. Rate-Limiting Steps in S Pathways

Sulfate assimilation is regulated by S status. When the amount of S in the plant is low, many enzymes involved in S acquisition and reduction are up-regulated, including sulfate permease, ATP sulfurylase and APS reductase (Gutierrez-Marcos et al., 1996; Takahashi et al., 2000). Expression of the gene encoding APS reductase is most closely correlated with S status and this enzyme is suspected to be a rate-controlling enzyme for the pathway (Leustek et al., 2000). There is also indication that ATP sulfurylase may be limiting for sulfate uptake and assimilation, because over-expression of the gene resulted in higher plant levels of both reduced and total S (Pilon-Smits et al., 1999). Another potentially limiting enzyme for Cys formation may be serine acetyltransferase, because over-expression in cytosol and plastids resulted in 3-fold and 6-fold higher Cys levels, respectively (Wirtz and Hell, 2003). The OAS thiol lyase protein is present at levels 2 orders of magnitude higher than serine acetyltransferase in chloroplasts (Droux et al., 1998) and hence not expected to be limiting. This is in agreement with the finding that transgenic plants that over-express OAS thiol lyase do not contain more Cys (Saito et al., 1994).

The regulatory mechanism for sulfate uptake and reduction to cysteine includes regulation at the transcriptional level in response to plant S status. When S limitation is sensed, up-regulation of several sulfate transporters as well as ATP sulfurylase and APS reductase occurs. The main shoot-to-root molecule that signals plant S status to the root appears to be GSH (Lappartient and Touraine, 1996; Lappartient et al., 1999), although Cys and intracellular sulfate have also been reported to control uptake of sulfate (Smith, 1975). When these compounds accumulate, S uptake and reduction are down-regulated. Another regulatory molecule is OAS, which up-regulates sulfate uptake and assimilation and can overrule internal S status (Neuenschwander et al., 1991; Smith et al., 1997).

Some regulation of Cys formation also occurs at the level of the two enzymes serine acetyltransferase and OAS thiol lyase (Bogdanova and Hell, 1997; Droux et al., 1998), which can exist as a complex as well as separately. When complexed with serine acetyltransferase, OAS thiol lyase does not synthesize Cys. On the other hand, serine acetyltransferase requires complexation with OAS thiol lyase to produce OAS. The formation of the complex is favored by sulfide, while OAS destabilizes the complex. Thus, when sulfide accumulates, serine acetyltransferase is activated and produces OAS, which then activates OAS thiol lyase. When the resulting reduced organic S compounds accumulate, the rates of further uptake and reduction of sulfate decrease.

The rate-controlling enzyme for Met synthesis from Cys is thought to be cystathionine-γ-synthase. Both cystathionine-γ-synthase and threonine synthase (TS) use OPH as a substrate, and the in vitro affinity for OPH is two orders of magnitude higher for threonine synthase than for cystathionine-γ-synthase (Curien et al., 1998; Ravanel et al., 1998b; Amir et al., 2002). If a similar affinity occurs in vivo, then most of the carbon flux would go towards threonine (Amir et al., 2002), making cystathionine-γ-synthase a likely rate-limiting step for Met synthesis. This hypothesis is supported by results from mutant and transgenic Arabidopsis and potato plants with reduced threonine synthase activity, which showed a substantial increase in Met levels and a small decrease in threonine levels, suggesting that reduced threonine synthase activity increases the flow of carbon towards Met (Bartlem et al., 2000; Zeh et al., 2001). Furthermore, mutant and transgenic Arabidopsis plants with increased cystathionine-γ-synthase activity accumulated up to 40 times more Met (Inba et al., 1994; Chiba et al., 1999; Suzuki et al., 2001; Hacham et al., 2002; J Kim et al., 2002). On the
other hand, antisense Arabidopsis plants with a 5- to 20-fold reduction in cystathionine-γ-synthase concentration showed no more than a 35% reduction in Met levels (Gakiere et al., 2000; J Kim and Leustek, 2000).

Neither cystathionine-γ-synthase nor cystathionine-β-lyase appear to be feed-back inhibited by end products at the protein level (Ravanel et al., 1998a,b). However, cystathionine-γ-synthase appears to be feed-back regulated by Met at the transcript level (Giovanelli et al., 1985). In A. thaliana, cystathionine-γ-synthase transcript levels were reduced by Met by autoregulation. A cystathionine-γ-synthase amino acid sequence encoded by exon-1 can act in cis to destabilize its own mRNA when activated by Met or one of its metabolites (Chiba et al., 1999). A regulatory role for cystathionine-γ-synthase rather than cystathionine-β-lyase is supported by the observation that overexpression of cystathionine-β-lyase does not enhance metabolic flux toward Met (Maimann et al., 2001).

The regulatory enzyme for GSH synthesis under unstressed conditions is thought to be γ-glutamylsynthetase (Noctor et al., 1996, 1998a,b). Under metal stress, γ-glutamylsynthetase activity is up-regulated both at the transcription level and the enzyme activity level, and GSH synthetase may become co-limiting (Zhu et al., 1999a). Since Cys levels are very low in cells, the supply of Cys is another potential limiting factor for GSH formation (Noctor et al., 1996). This limitation may be alleviated to some extent by metal-induced up-regulation of sulfate uptake and assimilation (Leustek et al., 2000; Nocito et al., 2002).

A substantial part of the synthesized Met is used for the formation of SAM. This molecule can act as an enzyme activator and thus influence metabolic fluxes through pathways. For instance, SAM activates threonine synthase, leading to enhanced threonine synthesis and reduced Met synthesis (Ravanel et al., 1998a). Also, as a precursor of the plant growth regulators ethylene and polyamines, SAM influences overall plant growth and development.

B. Regulation of S Metabolism in Response to the Environment

As mentioned above, S limitation induces sulfate uptake and assimilation at the transcriptional level, with GSH as an important signal molecule. While uptake and reduction of S are enhanced under S limitation, the synthesis of secondary S compounds (e.g. sulfation) is down-regulated, and secondary S compounds such as glucosinolates are even broken down to provide S for essential compounds (Maruyama-Nakashita et al., 2003). Sulfur limitation also affects the expression of seed storage proteins (H Kim et al., 1999), the rate of photosynthesis (Wykoff et al., 1998) and protein turnover (Gilbert et al., 1997). Conversely, when photosynthesis is reduced, sulfate assimilation is reduced as well. Accumulation of AMP and ADP were reported to inhibit ATP sulfurylase (Schwenn and Depka, 1977), offering a partial explanation of the mechanism involved.

The S assimilation pathway appears to also be developmentally regulated, and to be most active in young tissues (Rotte and Leustek, 2000), perhaps because in this time of rapid growth most reduced S is needed for protein synthesis. Light also stimulates S assimilation, as more reduced Fd is available for S reduction.

The S assimilation pathway is also regulated in coordination with nitrogen (N) assimilation and the ratio of reduced S to reduced N is typically maintained at 1:20 (Buchanan et al., 2000). Reduced S compounds activate the key enzyme of N reduction, nitrate reductase. Similarly, reduced N compounds stimulate the key enzymes of S reduction, ATP sulfurylase (Reuveny et al., 1980) and APS reductase (Koprivova et al., 2000) at the transcription level. The same reduced S and N compounds feed-back inhibit the key enzymes of their own biosynthetic pathways. Stimulation of S uptake and assimilation under low-S conditions does not happen when N is limiting, suggesting there is an N compound necessary for de-repression of S pathway enzymes (Yamaguchi et al., 1999).

Sulfur deprivation leads to oxidative stress due to a lack of GSH. This was demonstrated by the observation that pathways involved in stress resistance were up-regulated under S limitation in Arabidopsis in a transcriptome profiling study (Maruyama-Nakashita et al., 2003). Under other conditions of oxidative stress such as the presence of heavy metals, there is an increased demand for reduced S compounds like GSH, Cys and PCs. Hence, genes involved in uptake and reduction of sulfate are up-regulated at the transcription level under these conditions (Leustek et al., 2000; Nocito et al., 2002), as are genes involved in formation of GSH and PCs (Xiang and Oliver, 1998).

The regulatory proteins involved in S sensing and signal transduction in higher plants are not known. In Chlamydomonas reinhardtii several genes (SAC1 to SAC3) were identified that are involved in sensing S deficiency and mediating many physiological responses (Davies et al., 1994). SAC1 encodes a membrane protein with similarity to sodium dicarboxylate transporters (Davies and Grossman, 1998; Ravina et al., 2002). SAC1-deficient mutants are unable to
sense S deficiency and lack the associated increases in S-associated enzyme activities (Ravina et al., 2002). \( SAC2 \) appears to regulate APS reductase activity post-transcriptionally (Ravina et al., 2002), while \( SAC3 \) encodes a putative serine-threonine kinase (Davies et al., 1999). In higher plants no homolog of \( SAC1 \) has been found, but there are 12 homologs of \( SAC3 \) (Ravina et al., 2002). Some of these proteins may be involved in regulating plant S metabolism. Transcriptome analysis in Arabidopsis under S deficiency also yielded a number of putative transcription factors that were up-regulated under S deficiency and may function in signaling cascades of stress response (Maruyama-Nakashita et al., 2003).

### V. Involvement of S Pathways in Metabolism of Other Oxyanions

There is abundant evidence that enzymes of the S pathway also metabolize analogs of the related element selenium (Se). All sulfate transporters tested can also transport selenate (Smith et al., 1995; Hawkesford, 2003). Sulfate transport is inhibited by sulfate, selenate, arsenate, chromate, molybdate, and tungstate, again indicating that sulfate transporters may be involved in transport of these related oxyanions (Wilson and Bandurski, 1958; Leustek, 1996). Indeed, over-expression of a type-1 sulfate transporter resulted in enhanced accumulation of Se, Cr, V and W (E.A.H. Pilon-Smits et al., unpublished results), indicating that sulfate permease mediates uptake of these oxyanions in vivo. ATP sulfurylase, the enzyme that activates sulfate by binding it to ATP, was also shown to react with selenate as well as molybdate in vitro (Wilson and Bandurski, 1958). Over-expression of ATP sulfurylase in plastids resulted in higher Se accumulation, more reduced organic Se, and higher Se tolerance (Pilon-Smits et al., 1999), demonstrating the involvement of this enzyme in selenate reduction in vivo. More recently, over-expression of ATP sulfurylase was shown to also result in enhanced accumulation of the oxyanions of As, Cr, Cu, Mo, V and W (Wangeline et al., 2004). It is not clear at this point whether the end-products of ATP and these other oxyanions are stable. Results from in vitro studies indicate that besides sulfate only selenate can be further metabolized by the sulfate reduction pathway (Wilson and Bandurski, 1958).

Over-expression of OAS thiol lyase did not affect Se accumulation or tolerance (de Souza et al., 2000b), probably because this enzyme is not rate-limiting for these processes, although it is likely involved in SeCys formation. Over-expression of cystathionine-\( \gamma \)-synthase in plastids led to enhanced formation of DMSe, the volatile form of Se analogous to DMS, suggesting this enzyme is involved in, and rate-limiting for, Se volatilization from SeCys (van Huysen et al., 2003). Knockout of Met methyltransferase abolished DMSe production in Arabidopsis (Tagmount et al., 2002), showing the involvement of this enzyme in Se volatilization. The analog of DMSP, dimethylselenon-propionate (DMSeP) was shown to be the precursor of volatile DMSe production (de Souza et al., 2000a).

Based on these results, the entire S assimilation and volatilization pathway appears to be able to use the Se analogs as substrates. The same may be true for most other enzymes in the S pathway. The activity of most S-related enzymes on the Se analogs of their substrates may in most cases be a non-functional but unavoidable side-effect. However, it is also feasible that in some cases these activities have a function in plants. Selenium volatilization may be a way to get rid of excess toxic Se. Another potential way to detoxify Se is the conversion of SeCys to alanine and selenide, thereby preventing non-specific incorporation of SeCys in proteins. Plants may indeed use this strategy, because a plastidic NifS-like protein from Arabidopsis was shown to have 300-fold higher in vitro activity with selenocysteine (SeCys lyase activity) than with Cys (Pilon-Smits et al., 2002). Furthermore, its over-expression enhanced Se tolerance and accumulation (Van Hoewyk et al., 2005). Expression of a related mouse NifS-like protein (a SeCys lyase) in Arabidopsis also resulted in enhanced Se accumulation, and either enhanced or reduced Se tolerance depending on the intracellular location of the SeCys lyase protein. In these cases, Se incorporation in proteins was reduced (Pilon et al., 2003).

Many organisms (e.g. mammals, bacteria and Chlamydomonas) have essential Se metabolism. These organisms need Se because enzymes such as glutathione peroxidase, iodothyronine deiodinase, and formate dehydrogenase require a SeCys in their active site for activity (Stadtman, 1990, 1996). In bacteria, Se is also an essential component of special tRNAs that have a Se-containing uracyl analog in the wobble position (Mihara et al., 2002). For essential Se metabolism, selenate is taken up and assimilated to SeCys by the sulfate reduction pathway described above. SeCys is then converted into selenide and alanine by SeCys lyase activity (Mihara et al., 1999, 2000, 2002). The selenide formed is the substrate for selenophosphate synthase.
VI. Transgenic Approaches to Study and Manipulate S Metabolism

As already mentioned above, in several instances transgenic approaches have been used successfully to study the involvement and rate-limitation of enzymes in the biochemical pathways of S and related oxyanions. This has given insight into fundamental biological processes but has also potential for breeding plants with favorable properties for human use. For instance, enhanced capacity of plants to extract S from soil may give higher yields on marginal soils, and may enhance plant biotic and abiotic stress resistance. Higher levels of S compounds may also give plants a better flavor, or higher medicinal value. Since S volatilization can influence weather, manipulation of S volatilization may be used to try to promote rain in dry areas. Because of the involvement of S metabolism in accumulation and tolerance of other trace elements (other oxyanions and thiol-bound metals), it may be possible to engineer plants with enhanced capacity to tolerate and accumulate these pollutants from the environment (phytoremediation) by means of manipulating S metabolism.

We will end with a short overview of results obtained so far using plant biotechnology of S metabolism. Via over-expression of sulfate transporters, plants were created that show higher accumulation of S, Se, Cr, V and W (E.A.H. Pilon-Smits and N. Terry, unpublished results). Over-expression of ATP sulfurylase in Indian mustard (Brassica juncea) resulted in higher accumulation of S, Se, As, Cr, Cu, Mo, V and W (Pilon-Smits et al., 1999; Wangelin et al., 2004). Over-expression of (bacterial) serine acetyltransferase in tobacco resulted in increased resistance to oxidative stress (Blaszczyk et al., 1999), while over-expression of OAS thiol lyase in tobacco increased stress resistance to H₂S and sulfite (Youssefian et al., 1993; Saito et al., 1994). Expression of a S-rich seed albumin led to higher Met levels in seeds and increased nutritional value (Molvig et al., 1997). Selenium volatilization and Se tolerance were enhanced by over-expressing cystathionine-γ-synthase or SeCys methyltransferase (van Huyzen et al., 2003; LeDuc et al., 2004). Enhanced Se tolerance and accumulation was achieved by over-expression of enzymes with SeCys lyase activity (Pilon et al., 2003). Over-expression of the GSH-synthesizing enzymes γ-glutamylcysteine synthetase and GSH synthetase resulted in enhanced metal tolerance and accumulation (Zhu et al., 1999a,b), while over-expression of GSH reductase resulted in increased resistance to oxidative stress in tobacco and poplar trees (Aono et al., 1993; Foyer et al., 1995).

Together these results obtained from manipulation of S metabolism in plants have rendered new information about the enzymes involved in S metabolism and their importance as rate-limiting steps in the various pathways. Also, some of the transgenics obtained may find uses in agriculture, horticulture or environmental cleanup of polluted sites.
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