Cysteine and cysteine-related signaling pathways in Arabidopsis thaliana

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ABSTRACT

Cysteine occupies a central position in plant metabolism because it is a reduced sulfur donor molecule involved in the synthesis of essential biomolecules and defense compounds. Moreover, cysteine *per se* and its derivative molecules play roles in the redox signaling of processes occurring in various cellular compartments. Cysteine is synthesized during the sulfate assimilation pathway via the incorporation of sulfide to O-acetylserine, catalyzed by O-acetylserine(thiol)lyase (OASTL). Plant cells contain OASTLs in the mitochondria, chloroplasts and cytosol, resulting in a complex array of isoforms and subcellular cysteine pools. In recent years, significant progress has been made in *Arabidopsis*, in determining the specific roles of the OASTLs, and the metabolites produced by them. Thus, the discovery of novel enzymatic activities of the less abundant, like DES1 with L-cysteine desulfhydrase activity and SCS with S-sulfocysteine synthase activity, has provided new perspectives on their roles, besides their metabolic functions. Thereby, the research has been demonstrated that cytosolic sulfide and chloroplastic S-sulfocysteine act as signaling molecules regulating autophagy and protecting the photosystems, respectively. In the cytosol, cysteine plays an essential role in plant immunity; in the mitochondria, this molecule plays a central role in the detoxification of cyanide, which is essential for root hair development and plant responses to pathogens.

Keywords:

β-cyanoalanine synthase, cysteine desulfhydrase, cyanide, O-acetylserine(thiol)lyase, plant development, plant immunity, redox signaling, S-sulfocysteine synthase, stress, sulfide

INTRODUCTION

Sulfur is a macronutrient essential for plant growth and development that constitutes 0.3-0.5% of the total dry weight of plants. The most abundant form of sulfur present in nature is sulfate that is taken up by plants, and reduced and assimilated into cysteine (Cys). Therefore, Cys is the first organic compound containing reduced sulfur synthesized by the plant (Takahashi et al., 2011). The importance of Cys in plants is defined not only by its role as an amino acid in proteins but also by its function as a precursor for a large number of essential bio-molecules (Figure 1). The thiol group of cysteine is susceptible to oxidation, rendering disulfide bridges, which are determinants of the structure and folding of proteins and, consequently, of their stability and function (Haag et al., 2012). In addition, some proteins may undergo reversible reduction / oxidation of these disulfide bridges as a mechanism of redox regulation of their functions (Buchanan and Balmer, 2005). Moreover, thiol groups of cysteines, often located in their active sites, are essential for catalysis in many enzymatic reactions, like for example, the papain-like cysteine proteases that have been implicated in numerous plant cell processes (Richau et al., 2012).

Another equally important characteristic of cysteine is that it is the precursor molecule from which numerous sulfur-containing metabolites that are necessary for the development of life are synthesized. Examples of such metabolites include the other proteinogenic amino acid methionine, vitamins, cofactors and Fe-S clusters (Droux, 2004; Van Hoewyk et al., 2008; Wirtz and Droux, 2005). Of particular interest is the antioxidant glutathione (GSH) (Figure 1), the tripeptide γ-glutamyl-cysteinyl-glycine, which is regarded as the major determinant of cellular redox homeostasis (Foyer and Noctor, 2011; Noctor et al., 2012). This property of GSH is based on the redox regulation of the thiol group of its containing cysteine. Similarly, other functions of GSH, such as its role in the plant response to adverse environmental conditions, can also be attributed to the high reactivity of its thiol group. Examples of such functions include heavy metal detoxification through the GSH-derived peptides phytochelatins (Mendoza-Cozatl et al., 2011; Rea, 2012), or xenobiotic detoxification by GSH-conjugation via GSH-S-transferases (Dixon et al., 2002). Glutathione has also been implicated in the plant defense response to pathogens, along with the many other defense compounds formed in response to different biotic stresses, for example camalexin and glucosinolates (Rausch and Wachter, 2005). Recently, these latter defense metabolites involved in insect protection have been demonstrated to be essential in

the plant response to microbial pathogens as well (Bednarek et al., 2009; Clay et al., 2009). In all of these bio-molecules, their sulfur moieties, which act as functional groups, are derived from Cys, indicating that their biosynthetic pathways are intimately linked. Another metabolite that is closely linked to cysteine is the hormone ethylene (Figure 1), which is involved in many aspects of the plant life cycle, including seed germination, root hair development, seedling growth, leaf and petal abscission, climacteric fruit ripening, organ senescence, and the modulation of plant responses to stresses. Ethylene is synthesized in the cytosol from methionine via S-adenosyl-L-methionine (SAM), which is converted to 1-aminocyclopropane-1-carboxylic acid (ACC), and ACC is converted to ethylene (Bleecker and Kende, 2000). For the reasons mentioned above, we can conclude that Cys occupies a central position in plant primary and secondary metabolism.

The final inorganic sulfur compound produced by the assimilatory reduction of sulfate by photosynthetic organisms is sulfide, which is then incorporated into an amino acid skeleton to form cysteine (Takahashi et al., 2011). The biosynthesis of Cys is accomplished by the sequential reaction of two enzymes (Figure 1): serine acetyltransferase (SAT), which synthesizes the intermediary product O-acetylserine (OAS) from acetyl-CoA and serine, and O-acetylserine(thiol)lyase (OASTL), which incorporates the sulfide to OAS, producing Cys, requiring pyridoxal-5'-phosphate as cofactor. Together, these enzymes form the hetero-oligomeric cysteine synthase complex, initially described in bacteria and later studied extensively in plants. Protein interactions within the complex strongly modify the kinetic properties of SAT, enabling this enzyme to more efficiently synthesize OAS. OASTL, by contrast, is active in its abundant free form and exhibits greatly reduced activity when in a complex with SAT (Droux et al., 1998; Wirtz and Hell, 2006). The formation of this complex, which provides an effective mechanism to modulate cysteine production, is dependent on the relative available amounts of sulfide and OAS, and has consequently been considered a sensor of the intracellular sulfur state of the plant (Yi et al., 2010).

Plant cells contain different SAT and OASTL enzymes depending on their location in the cytosol, plastid or mitochondrion, resulting in the presence of a variety of isoforms and different subcellular Cys pools. The model organism *Arabidopsis thaliana* is the most widely investigated plant, and five *SAT* (Howarth et al., 2003) and nine *OASTL* genes (Wirtz et al., 2004) have been identified in its genome. An *in silico* search for gene orthologs in various photosynthetic organisms indicates the presence of multiple *SAT* and *OASTL* orthologs, suggesting their organization to be similar to that of *A. thaliana* (Table 1). Additionally, it is evident that there is

no agreement among the numbers of *SAT* and *OASTL* genes within the same species, as there is always a higher proportion of *OASTL* genes, even in simple organisms such as moss or unicellular algae. This observation calls into question whether all OASTL enzymes form complexes with their SAT partners, or if instead, some OASTL enzymes have functions outside of an involvement in the primary cysteine biosynthesis pathway, as has been demonstrated in *Arabidopsis*.

In *A. thaliana,* the most abundant *OASTL* genes at the transcriptional level encode the cytosolic OAS-A1 (At4g14880), the plastidial OAS-B (At2g43750) and the mitochondrial OAS-C (At3g59760) isoforms. These proteins are considered authentic OASTLs because they catalyze the synthesis of cysteine from OAS and sulfide (Table 2). Another property that defines a true OASTL is the ability to interact with SAT, which has been demonstrated using various approaches (Bonner et al., 2005; Heeg et al., 2008; Jez and Dey, 2013). Very recently, it has been reported that at least a functional one of these major OASTLs in the pollen is required for the successful fertilization (Birke et al., 2013). The OASTL enzyme family includes an additional highly expressed isoform located in the mitochondria, CYS-C1 (At3g61440), that actually functions as a β-cyanoalanine synthase (CAS) (Hatzfeld et al., 2000; Yamaguchi et al., 2000). This enzyme catalyzes the conversion of cysteine and cyanide to hydrogen sulfide and β-cyanoalanine, and plays an essential role in the detoxification of cyanide in the mitochondria for the maintenance of appropriate levels of cyanide for signaling in specific plant processes (Garcia et al., 2010), as described below. One of the *OASTL* genes, *OAS-A2*, does not produce a functional protein due to the presence of an in-frame stop codon and an unspliced intron within its gene sequence.

The remaining OASTL proteins, the cytosolic CYS-D1 (At3g04940), CYS-D2 (At5g28020) and DES1 (At5g28030) and the plastidic CS26 (At3g03630), all expressed at considerably lower levels than the aforementioned proteins, were identified by sequence homology upon completion of the *A. thaliana* genome sequence. These proteins are thought to have auxiliary functions with respect to the major isoforms. Recent investigations of DES1 and CS26, however, have shed light on their functions and the enzymatic reactions they catalyze (Gotor et al., 2010). DES1 is a novel L-cysteine desulfhydrase located in the cytosol that catalyzes the desulfuration of cysteine to sulfide plus ammonia and pyruvate (Table 2) (Alvarez et al., 2010). This enzyme is essential in regulating the homeostasis of cysteine, as well as in modulating the generation of sulfide in the cytosol for signaling purposes (Alvarez et al., 2012b; Gotor et al., 2013; Romero et al., 2013), as

described later. Meanwhile, CS26 exhibits S-sulfocysteine synthase activity and catalyzes the incorporation of thiosulfate to OAS to form S-sulfocysteine, and we propose in this review to rename as SCS (Table 2). This activity, which plays an important role in chloroplast function, was recently discovered for the first time in plants, although it had previously been reported in bacteria (Bermudez et al., 2010). This enzyme appears to be essential to proper photosynthetic performance under long-day growth conditions and is thought to act as a sensor of the chloroplast redox status (Bermudez et al., 2012; Gotor and Romero, 2013), as described below.

In the following sections, we will describe the current knowledge concerning the importance of cysteine and its derivative molecules in *Arabidopsis thaliana*. With a focus on separate cell compartments, we will unravel the role of these compounds in both the signaling and control of different plant processes.

CYSTEINE IN THE CYTOSOL

Independent investigations of the *Arabidopsis OASTL* and *SAT* gene families have demonstrated that the cytosol is the major site of cysteine synthesis (Haas et al., 2008; Heeg et al., 2008; Lopez-Martin et al., 2008a; Watanabe et al., 2008a; Watanabe et al., 2008b). Cysteine concentrations in the cytosol are estimated to be greater than 300 μM, whereas the other cell compartments each contain less than 10 μM cysteine (Krueger et al., 2009). Therefore, the cytosolic OASTL isoform OAS-A1 is the major contributor in cysteine biosynthesis (Figure 2). Cysteine can, however, be a very toxic molecule when present at concentrations above a certain threshold, as a result of its high reactivity. Thiols are easily oxidized to form species with sulfur in higher oxidation states, which subsequently inhibit enzyme activity; furthermore, cysteine reduces ferric iron at an exceptional high rate and promotes oxidative damage through the Fenton reaction (Jacob et al., 2003; Park and Imlay, 2003). Consequently, cysteine homeostasis must be precisely maintained in the cytosol. The recent identification of DES1 as a novel L-cysteine desulfhydrase (Alvarez et al., 2010), which catalyzes the desulfuration of L-cysteine to produce sulfide plus ammonia and pyruvate, (Figure 2) has led us to propose that the maintenance of cysteine homeostasis occurs via the coordination of OAS-A1 and DES1 enzymatic activities throughout the life cycle of the plant.

Our suggestion of the coordinated functions of OAS-A1 and DES1, and the subsequent impact of this connection on plant metabolism, is based on the phenotypes of their corresponding null

mutants. Biochemical characterization of the T-DNA insertion mutants *oas-a1* and *des1* reveals that the total intracellular cysteine concentration is reduced by approximately 35% in the *oas-a1* mutant and increased by approximately 25% in the *des1* mutant, relative to the wild type. It is plausible to think that the range of change of 35-25 % of the total intracellular cysteine is the maximum possible not to reach the toxic threshold. Considering the enzymatic reactions catalyzed by OAS-A1 and DES1, these observations were expected (Figure 2). Interestingly, the *oas-a1* mutant plants are oxidatively stressed as a result of an imbalance between the generation and removal of ROS (Lopez-Martin et al., 2008a), and accordingly, the *des1* mutant plants show enhanced antioxidant defenses, indicated by a significant decrease in ROS production (Alvarez et al., 2010). These characteristic phenotypes can be observed *in vivo* in plant tissues by monitoring the production of hydrogen peroxide, observing a significant ROS production in roots of *oas-a1* mutant, which is not detectable in either wild type or *des1* roots. Moreover, H_2O_2 is a signaling molecule in programmed cell death (Dat et al., 2003), and patches of dead cells are visible in the leaves of *oas-a1* mutant that are not observable in the wild type or *des1* mutant leaves. Altogether, these findings indicate that cysteine is an important determinant of the antioxidative capacity of the cytosol in *Arabidopsis* (Alvarez et al., 2010; Lopez-Martin et al., 2008a; Lopez-Martin et al., 2008b). Consequently, cysteine levels in the cytosol should influence all plant processes in which redox signaling plays a regulatory role. Thus, the mutation in *DES1* leads to premature leaf senescence as is visible when *des1* mutant plants are grown side by side with wild type plants (Figure 3). This early senescence is also evidenced at the cellular and transcriptional levels. The *des1* mutant shows increased expression of senescence-associated genes such as *SAG12*, *NAP* and *PR1*, a transcriptional profile that is generally compatible with premature senescence, as well as an accumulation of *de novo* senescence-associated vacuoles (SAVs) in mesophyll cells. By contrast, transcriptomic analysis of the *oas-a1* mutant shows significantly reduced expression of senescence-associated genes such as *SAG12* and *SEN1*; furthermore, the presence of SAVs is undetectable (Alvarez et al., 2012a; Alvarez et al., 2010; Lopez-Martin et al., 2008a).

The statement that OAS-A1 and DES1 have opposing functions with regards to regulating cytosolic cysteine for redox signaling is further corroborated by the observation of opposing phenotypes in the null mutants upon exposure to adverse conditions. Under abiotic stress conditions such as Cd stress, the *oas-a1* mutant shows marked sensitivity to the presence of Cd, indicated by its compromised viability at concentrations as low as 75 to 100 μM (Lopez-Martin

et al., 2008a). On the contrary, the *des1* mutant shows a significantly increased tolerance to Cd, compared to wild type plants, at the high concentration of 250 μM (Alvarez et al., 2010). Thus, levels of cysteine in the cytosol profoundly affect the plant's defense responses to abiotic stresses, and it is evident that increased cysteine to levels below the toxic threshold, induce stress tolerance. These results were previously observed when OAS-A1 was over-expressed in *Arabidopsis*, demonstrating that increased cysteine availability is responsible for enhanced cadmium tolerance and accumulation (Dominguez-Solis et al., 2001; Dominguez-Solis et al., 2004).

Analysis of the responses of the *oas-a1* and *des1* mutants to pathogen attack confirm, once again, the essential role of cysteine homeostasis in the cytosol, and the involvement of these two enzymes (OAS-A1 and DES1) in the control of this homeostasis (Alvarez et al., 2012a). In fact, these mutants show an altered basal resistance to pathogens, such that increased cytosolic cysteine content (observed in the *des1* mutant) is associated with enhanced resistance to pathogens, whereas decreased cytosolic cysteine content (observed in the *oas-a1* mutant) is associated with decreased resistance to pathogens. In the case of the *des1* mutant, its behavior resembles that of constitutive systemic acquired resistance mutants, characterized by high resistance to biotrophic and necrotrophic pathogens, salicylic acid (SA) accumulation and *WRKY54* and *PR1* induction. On the contrary, the *oas-a1* mutant is more sensitive to both types of pathogens, displays indistinguishable SA levels and induction of *PR1*of those of wild type and several *WRKY* genes are repressed. Moreover, the *oas-a1* mutant lacks the hypersensitive response (HR) associated with effector-triggered immunity, suggesting that cytosolic cysteine has an essential role in the initiation of the HR response (Alvarez et al., 2012a). An independent investigation has also confirmed the existence of an interaction between OAS-A1 and components of plant immunity, which highlights the emerging role of cysteine metabolism in plant immunity (Tahir et al., 2013).

In conclusion, accurate regulation of cytosolic cysteine homeostasis by the action of OAS-A1 and DES1 is critical for plant metabolism and stress responses. Thus, internal factors such as developmental stage, and environmental factors such as abiotic and biotic stress influence the ratio of cysteine / sulfide levels through the modulation of the activities of these two enzymes.

Post-translational modification represents an important level of regulation of protein function in all living organisms, and is being increasingly investigated. A proteomic analysis revealed that the major cytosolic and plastidic OAS-TLs, OAS-A1 and OAS-B, are subject to N-terminal

acetylation (Wirtz et al., 2010). Further, phosphorylation of the plastidic SAT isoform from soybean has been reported (Liu et al., 2006); and in *Arabidopsis* it has been suggested for a cytosolic low abundant SAT isoform and also for the major OAS-A1, by interaction with 14-3-3-proteins (Shin et al., 2011). Among the different post-translational modifications that have been characterized, those mediated by the action of nitric oxide (NO)-derived modifiers have recently attracted the attention of many plant biology researchers (Begara-Morales et al., 2013; Corpas and Barroso, 2013). A well-documented protein modification of a member of the OASTL family is the nitration of Tyr residues on the OAS-A1 protein, which modulates its activity (Alvarez et al., 2011). Nitration of Tyr residues under physiological conditions is largely the result of the protein interacting with the strong nitrating agent peroxynitrite, which is formed by the reaction of NO with a superoxide anion (Szabo et al., 2007). After exposure to peroxynitrite, OAS-A1 is markedly more sensitive to nitration than the other OASTLs, leading to inhibition of this enzyme's activity. Inhibition of OAS-A1 activity is a result of the specific nitration of the Tyr302 residue, which drastically reduces both binding of the O-acetylserine substrate to the nitrated protein and stabilization of the pyridoxal-5'-phosphate cofactor through hydrogen bonds (Alvarez et al., 2011). Therefore, post-translational modification of OAS-A1 by Y-nitration may represent a rapid and efficient regulatory mechanism for controlling cysteine homeostasis in the cytosol under stress conditions that often lead to the production of reactive oxygen and nitrogen species.

In recent years, emerging experimental evidence from numerous plant biology studies has shown H_2S to be a signaling molecule of equal importance to NO and H_2O_2, an observation that is consistent with data previously shown in animal systems. In plants, H_2S has been implicated in the protection against metal stress (Zhang et al., 2008; Zhang et al., 2010), and the regulation of photosynthesis (Chen et al., 2011) and stomatal movement (Garcia-Mata and Lamattina, 2010; Lisjak et al., 2010), for example. H_2S is endogenously produced in mammalian tissues by enzymatic reactions of L-Cys, primarily via two cytoplasmic enzymes, cystathionine-γ-lyase and cystathionine-β-synthase, which both require pyridoxal-5'-phosphate as a cofactor. Ammonia and pyruvate are by-products of these reactions (Gadalla and Snyder, 2010; Wang, 2012). DES1 was found to have L-cysteine desulfhydrase activity, also dependent on pyridoxal-5'-phosphate, in the cytosol of *Arabidopsis* (Alvarez et al., 2010). To our knowledge, DES1 is the only enzyme that has an unequivocally established involvement in the degradation of cysteine and the concomitant generation of hydrogen sulfide in the plant cytosol. Accordingly, we conclude that DES1 is responsible for modulating the generation of sulfide for signaling purposes in the plant cell

(Romero et al., 2013). Uncertainty regarding this role could arise from the fact that in plants, the chloroplast is the main source of sulfide via sulfate reduction in the sulfur assimilation pathway (Takahashi et al., 2011). Although it has been proposed that H_2S reaches the cytosol via diffusion through the chloroplast envelope membrane, hydrogen sulfide is weakly acidic and dissociates in aqueous solution into H^+ and SH^-, and this ionized form cannot permeate membranes (Kabil and Banerjee, 2010). Thus, at the pH of 8.5 that is maintained in the chloroplast stroma under illumination, sulfide is mainly present in its charged form and is therefore unable to be transported across the chloroplast envelope. Therefore, DES1 modulates the generation of the signaling molecule sulfide in the plant cytosol, irrespective of nutrient conditions (Romero et al., 2013).

Recent research in our lab has demonstrated that sulfide exerts a general effect on autophagy in plants through negative regulation of this process (Gotor et al., 2013). Autophagy is a universal mechanism with a pro-survival role in eukaryotic cells, involving the digestion of cell contents to recycle necessary nutrients or to degrade damaged or toxic components. The most important feature of autophagy is the *de novo* synthesis of double membrane-bound structures called autophagosomes, which engulf and deliver materials to the vacuole for breakdown. Proteins involved in the autophagy process (ATG proteins) have been used to monitor autophagic activity in plants, the most commonly used protein being ATG8, which is tethered to the autophagosomes by lipidation (recent reviews of (Li and Vierstra, 2012; Perez-Perez et al., 2012). The detailed characterization of *des1* mutant has provided insight into the role of the sulfide generated from Cys in the cytosol as a signaling molecule regulating the process of autophagy. Mutations of the *DES1* gene impede H_2S generation in the cytosol and lead to the accumulation and lipidation of ATG8 isoforms in *Arabidopsis*, a landmark of autophagy activation. Restoration of the capacity of H_2S generation eliminates phenotypic differences between the null mutants and the wild type plants, thereby reversing the autophagy activation. Interestingly, exogenous sulfide is also able to suppress the induction of autophagy caused by carbon starvation in wild type plants, whereas exogenous ammonium, a second product of DES1 activity, has no effect on autophagy (Alvarez et al., 2012b). In conclusion, DES1 is responsible for the generation of sulfide in the cytosol, which subsequently behaves as a signaling molecule, acting as a repressor of autophagy.

CYSTEINE IN THE MITOCHONDRION

In *Arabidopsis*, both by the specific down-regulation of mitochondrial SAT by iRNA and by employing T-DNA insertion mutants of members of the SAT family, it has been demonstrated that mitochondria, not chloroplasts or the cytosol, are the dominant source of OAS *in vivo* for the bulk of cysteine synthesis in the cytosol (Haas et al., 2008; Krueger et al., 2009; Watanabe et al., 2008b). Therefore, little attention has been given to the OASTL family members responsible for cysteine synthesis in the mitochondria. In *A. thaliana*, two highly expressed proteins exist in the mitochondria, the true OASTL, OAS-C, which catalyzes the incorporation of sulfide to O-acetylserine to produce cysteine, and the β-cyanoalanine synthase, CAS-C1 (former CYS-C1), which catalyzes the conversion of cysteine and cyanide to hydrogen sulfide and β-cyanoalanine (Watanabe et al., 2008a) (Figure 2). Mitochondrial OAS-C contributes only 5% to the total OASTL activity, but it has been suggested that OAS-C plays a much more important role than previously assumed (Heeg et al., 2008). Recent results have allowed us to propose that the significance of both enzymes, OAS-C and CAS-C1, is related to their roles in proper sulfide and cyanide detoxification in the mitochondria (Alvarez et al., 2012c; Garcia et al., 2010). However, an independent investigation has suggested that the biosynthesis of cysteine by OAS-C in mitochondria contributes to the sulfide detoxification along with a novel mechanism independent of OASTL. In addition, the activity of OAS-C is not required for cyanide detoxification (Birke et al., 2012).

In non-cyanogenic species, such as *A. thaliana*, the main source of cyanide is derived from the biosynthesis of the phytohormone ethylene and the phytoalexin camalexin. The first committed step of ethylene biosynthesis is the conversion of S-AdoMet to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase. ACC is then oxidized by ACC oxidase to form ethylene and cyanoformic acid, which is spontaneously degraded giving carbon dioxide and cyanide (Bleecker and Kende, 2000; Peiser et al., 1984). During the biosynthesis of camalexin, the tryptophan-derived intermediate indole-3-acetonitrile is conjugated with cysteine to act as a substrate for the cytochrome P450 enzyme CYP71B15. This enzyme catalyzes the formation of the thiazoline ring, as well as the release of cyanide and the subsequent oxidative decarboxylation of dihydrocamalexic acid to camalexin (Bottcher et al., 2009). Therefore, under certain developmental or environmental conditions, plant produce significant amounts of cyanide that may be harmful to their cells. In mitochondria, cyanide is a potent inhibitor of cytochrome c oxidase, which constitutes complex IV of the mitochondrial respiratory chain (Cooper and Brown, 2008); therefore, cyanide accumulation must be prevented. The main cyanide detoxification

process described in plants is the conversion of cyanide to β-cyanoalanine, which is then converted to Asn, Asp, and ammonia by NIT4 class nitrilases, thus allowing for the recycling of nitrogen within the plant (Piotrowski, 2008).

The mitochondrial CAS-C1 is primarily involved in the adequate detoxification of cyanide through the formation of β-cyanoalanine; however, a co-product of this CAS enzymatic activity is hydrogen sulfide, which also inhibits oxygen consumption via inhibition of the mitochondrial cytochrome c oxidase. In mammalian systems, both HCN and H_2S are non-competitive inhibitors, with respect to oxygen, and show similar inhibition constants (Cooper and Brown, 2008). Accordingly, hydrogen sulfide produced during the detoxification of cyanide must be detoxified in the mitochondria by the authentic OASTL, OAS-C, which produces cysteine. Cysteine, in turn, could be used by CAS-C1 to detoxify cyanide, thus generating a cyclic pathway for cyanide detoxification in the mitochondria (Figure 2). Consequently, both CAS-C1 and OAS-C, by acting jointly, play an essential role in the modulation of cyanide levels in the mitochondria. This suggestion is reinforced by the similarity in the phenotypic characteristics of the corresponding null mutants (Alvarez et al., 2012c; Garcia et al., 2010).

The loss of either a functional CAS-C1, or a functional OAS-C causes a significant accumulation of cyanide, in comparison to wild type plants, which is mainly observable in root tissues of plants, even under controlled growth conditions where ethylene production is low. Moreover, an increase in the alternative oxidase (AOX) pathway, as demonstrated by an increase in *AOX1A* gene expression in the mutants compared to the wild type is also detected. Plant mitochondria possess two different pathways of electron transport at the ubiquinone level: the cyanide- and sulfide-sensitive cytochrome pathway, and the resistant alternative pathway. The alternative oxidase is responsible for the latter and is not coupled to ATP synthesis, as is the case in the cytochrome pathway (Vanlerberghe and McIntosh, 1997). Therefore, the cyanide produced in the *cas-c1* mutant uncouples the respiratory electron chain dependent on the cytochrome pathway and this uncoupling induces the AOX activity. The cyanide accumulation in the *cas-c1* mutant plants correlates with very interesting phenotypic characteristics. On one hand, a defect in root hair formation (Garcia et al., 2010), and on the other hand, an altered response to plant pathogens (Garcia et al., 2013).

Phenotypic analysis of soil-grown *cas-c1* plants reveals no apparent change in the development and growth of the aerial part of the plant. However, when the mutant is grown on vertical plates, we clearly observe that the root hairs begin to grow out and away from the root

surface but do not elongate to form normal hairs (Garcia et al., 2010). The loss of a functional OAS-C results in phenotypic characteristics very similar to those given by the loss of the CAS-C1 enzyme, indicating that the *oas-c* null mutant is defective in root hair elongation (Alvarez et al., 2012c) (Figure 4). In both cases, genetic complementation of the *cas-c1* and *oas-c* mull mutants with the corresponding *CAS-C1* and *OAS-C* genes rescues the impairment of root hair elongation and restores the wild type phenotype, thus confirming that the observed phenotypes were indeed due to the mutations (Alvarez et al., 2012c; Garcia et al., 2010). Furthermore, the root hair defect is phenocopied in wild type plants by the exogenous addition of cyanide to the growth medium and is reversed by the addition of hydroxocobalamin, the most commonly used antidote for severe acute cyanide poisoning in humans (Garcia et al., 2010). In conclusion, our research has demonstrated that discrete accumulation of cyanide is not toxic to the plant but acts as a strong inhibitor of root hair elongation, and that the mitochondrial CAS-C1 and OAS-C are essential in maintaining a low enough level of cyanide for proper root hair development to occur.

Related to plant-pathogen interaction, *cas-c1* plants present an increased susceptibility to the necrotrophic fungus *Botrytis cinerea* and an increased tolerance to the biotrophic *Pseudomonas syringae* pv *tomato* DC3000 bacterium and *Beet curly top virus* (Garcia et al., 2013). This altered response is completely dependent on cyanide, as demonstrated by genetic complementation or by treatment with the antidote hydroxocobalamin. In addition, the transcriptional regulation of the *CAS-C1* gene during the three plant-pathogen interactions analyzed allows a differential accumulation of cyanide in each interaction, suggesting that CAS-C1 is involved in the signaling pathway, leading to resistance or sensitivity depending on the type of pathogen. Furthermore, cyanide accumulation and *CAS-C1* gene expression are negatively correlated during compatible and incompatible plant-bacteria interactions (Garcia et al., 2013).

All these data suggest that cyanide, a low-M_r and highly hydrophilic molecule acts as a signal in plants. In roots, the mechanism by which the cyanide molecule inhibits the elongation of root hairs is currently unknown, although several hypotheses can be made. For example, cyanide could trigger a repressing signaling pathway, generated in the mitochondria, that regulates the plasma membrane NADPH oxidase, an enzyme necessary for the establishment of the tip-based Ca^{2+} gradient, which is in turn essential for polar growth in root hair (Foreman et al., 2003). In the regulation of the plant immune responses, we hypothesize that cyanide could uncouple the respiratory electron chain dependent on the cytochrome c oxidase, and this uncoupling may induce the alternative oxidase activity and the accumulation of ROS, which would act by

stimulating the salicylic acid-dependent signaling pathway of the plant immune system.

CYSTEINE IN THE CHLOROPLAST

Arabidopsis chloroplasts contain two OASTL isoforms, OAS-B and SCS, which resemble the isoforms present in bacteria, encoded by the *cysK* and *cysM* genes, respectively (Byrne et al., 1988; Hulanicka et al., 1986). The enzymes encoded by *cysK* and *OAS-B* are OASTL isoforms that catalyze the incorporation of sulfide to form cysteine; the enzymes encoded by *cysM* and *SCS* are S-sulfocysteine synthases that catalyze the incorporation of thiosulfate into OAS to form S-sulfocysteine (S-Cys) (Figure 2) (Bermúdez et al., 2012; Bermudez et al., 2010; Nakamura et al., 1984). In photosynthetic organisms, sulfate reduction occurs only in the plastid because the reductive steps catalyzed by adenosine 5'-phosphosulfate reductase and sulfite reductase are restricted to this compartment (Takahashi et al., 2011); however, according to data from knock-out lines, OAS-B is not the main contributor to the total OASTL activity in leaves, as plants lacking this protein show no apparent phenotype when grown under long- or short-day photoperiods (Bermudez et al., 2010; Heeg et al., 2008; Watanabe et al., 2008a). This scene changes in the case of the enzyme SCS which, despite being a minor isoform, plays an essential role in chloroplast redox control (Bermudez et al., 2010). Detailed characterization of null mutants reveals that the loss of SCS function results in phenotypic differences depending on the light regime (Figure 5). Under long-day conditions (LD) the *scs* mutant plant exhibits a significant reduction in size and leaf paleness; by contrast, under short-day conditions (SD), the *scs* mutant plants are indistinguishable from the wild type plants. Genetic complementation of the null mutant with the *SCS* gene restores the phenotype of the mutant plants grown under LD conditions, thus verifying that the observed phenotype of the *scs* mutant is indeed due to the disruption of the *SCS* gene (Bermudez et al., 2010). Interestingly, accumulation of ROS, such as superoxide radicals and hydrogen peroxide, is detected in leaves of the *scs* mutant plants, observable under LD conditions (Bermudez et al., 2010).

Photosynthetic characterization of the *scs* mutant under long-day growth conditions reveals significant reductions in most photosynthetic parameters, including net CO_2 assimilation rate, mesophyll conductance, and mitochondrial respiration in darkness. However, under short-day growth conditions, the *scs* mutant behaves similarly to wild type, in terms of photosynthetic performance (Bermúdez et al., 2012). The mutant phenotype and the photosynthetic rates

observed in *scs* plants grown under LD but not SD conditions are indicative of severe damage to the photosynthetic machinery, resulting from failure of the photosystem repair mechanisms during periods of prolonged exposure to light. Because adaptation to high light requires that plants sense the light conditions and modify their transcript profile and metabolism to balance the production and detoxification of ROS (Lepisto and Rintamaki, 2012), the absence of S-sulfocysteine synthase activity in the chloroplast disrupts this balance and prevents the plant from defending itself against photochemical damage to the photosystems and to the electron transport chain under a LD photoperiod. Therefore, under low light, the *scs* mutant behaves as through it were growing under high light conditions (Bermúdez et al., 2012).

The OAS-B and SCS proteins have two important features that render them functionally different from one another. First, the SCS protein is located in the lumen, in contrast to the OAS-B isoform, which is located in the stroma (Bermúdez et al., 2012). Second, the SCS protein has S-sulfocysteine synthase activity, incorporating thiosulfate instead of sulfide, which is incorporated by the stromal isoform (Bermudez et al., 2010) (Figure 2). Based on current knowledge, we suggest that S-sulfocysteine synthase functions as a protein sensor in the thylakoid lumen in the following way: under conditions of excess light, the light absorbed by the chloroplasts exceeds their photosynthetic capacity and can lead to the production of ROS, which may either interfere with the reduction of sulfite, or oxidize sulfide to form thiosulfate. SCS detects the accumulation of thiosulfate and generates the S-sulfocysteine molecule that triggers protection mechanisms in the photosynthetic apparatus, acting as a mild oxidant in the lumen (Gotor and Romero, 2013).

Despite their functional differences, OAS-B and SCS have something important in common: both depend on the same substrate, OAS, to incorporate sulfide or thiosulfate, respectively, and therefore compete for this substrate. Considering that the protein OAS-B is significantly more abundant than SCS, the availability of OAS for the synthesis of S-sulfocysteine in the lumen is likely limited by OAS-B activity within the stroma. *Arabidopsis* contains five SAT isoforms localized in the cytosol, mitochondrion and plastid, which are responsible for the synthesis of OAS. Analysis of single or multiple mutant combinations lacking SAT isoforms provides evidence that OAS can either diffuse, or be transported into and out of these organelles (Krueger et al., 2009; Watanabe et al., 2010). In this manner, quadruple SAT mutants whose total SAT activity is reduced up to 9% of the wild type level display a wild type-like growth phenotype. However, quadruple SAT mutants with less than 5% SAT activity show retardation of plant

growth, slight chlorosis and reduced chlorophyll content, thus resembling the phenotype of the *scs* mutant (Watanabe et al., 2010). Interestingly, the chloroplastic SAT isoform has been shown to interact with the cyclophilin CYP20-3, which may mediate the association of SAT and OAS-B to form the cysteine synthase complex within the chloroplast for OAS synthesis (Dominguez-Solis et al., 2008). The *cyp20-3* mutant shows reduced cysteine synthesis and a severe stress phenotype under light or stress conditions; however, single *oas-b* or *sat* mutants are not sensitive to such stress conditions, as would be expected for a phenotype that is dependent on cysteine levels (Bermudez et al., 2010; Dominguez-Solis et al., 2008; Watanabe et al., 2008b). Furthermore, the phenotype of *cyp20-3* resembles the observed in the *scs* mutant, suggesting that S-sulfocysteine synthesis and redox regulation within the lumen may be compromised in *cyp20-3*; however, this point has not been studied.

CONCLUDING REMARKS

The enzymes belonging to the OASTL family have an essential role in plant metabolism and have traditionally been studied in the context of their involvement in the primary sulfate assimilation pathway. The main focus of previous research has been placed on the most abundant and authentic OASTL enzymes, whereas less attention has been given to the other enzymes that have different activities of cysteine biosynthesis. Such is the case for the minor enzymes, cytosolic DES1 with L-cysteine desulfhydrase activity and chloroplastic SCS with S-sulfocysteine synthase activity, as well as for the major mitochondrial enzyme CAS-C1 with β-cyanoalanine synthase activity. Recent investigation of these proteins highlights the importance of cysteine and several of its related molecules, such as sulfide, S-sulfocysteine and cyanide, as signaling molecules involved in regulating essential processes in the plant, such as photosynthesis, plant protection against adverse conditions, plant immunity, autophagy, and root development (Figure 6). Therefore, it is fundamental a change of concept for cysteine, from playing a mere role in metabolic function to a new concept of cysteine and related molecules performing signaling roles. This novel view of the metabolism of cysteine reveals new and very interesting areas for potential investigation, such as the specific function, targets, and regulation of these molecules involved in the signaling and control of different plant processes, as well as the mechanisms underlying.

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Figure Legends

Figure 1. Enzymatic reactions involved in the biosynthesis of cysteine and overview of the roles of cysteine in plant metabolism.

Figure 2. Subcellular localization of the different members of the OASTL enzyme family and reactions catalyzed by them in *A. thaliana***.**

Figure 3. Early-senescence phenotype of the *des1* mutant plants.
Wild-type Columbia-0 (Col-0) and *des1-1* mutant plants were grown side by side in soil for 4 weeks under long-day photoperiod (16h light/8h dark) and nutrient-sufficient conditions. A representative image is shown.

Figure 4. Root phenotype of the *cas-c1* and *oas-c* mutants.
Upper panel: Bright field images of 2-week-old wild type and *cas-c1* mutant seedlings, growing on vertical MS medium (Garcia et al., 2010) (www. plantcell.org; copyright American Society of Plant Biologists). Lower panel: Bright field images of roots from wild type and *oas-c* mutant seedlings, growing for 8 d on vertical MS medium (Alvarez et al., 2012c).

Figure 5. Phenotypes of the *scs* and *oas-b* mutants and the complemented *scs* line.
Plants grown on soil under long-day (LD, 16h light/8h dark) and short-day (SD, 8h dark/16h light) conditions for 3 and 4 weeks, respectively. Details in (Bermudez et al., 2010) (www. plantcell.org; copyright American Society of Plant Biologists).

Figure 6. Processes regulated by cysteine and cysteine-related molecules in the *Arabidopsis* cell.

Table 1. Number of *SAT* and *OASTL* gene orthologs in photosynthetic organisms.

Organism	***SATs***	***OASTLs***
Chlamydomonas reinhardtii	2	4
Physcomitrella patens	4	3
Arabidopsis thaliana	5	9
Brachypodium distachyon	4	8
Carica papaya	3	7
Glycine max	11	18
Medicago truncatula	1	14
Oryza sativa	6	13
Populus trichocarpa	4	12
Selaginella moellendorfii	3	4
Sorghum bicolor	4	8
Vitis vinifera	5	10
Zea mays	5	11

The ortholog search was performed using the Phytozome v6.0 database (www.phytozome.org)

Table 2. OASTL gene family in *Arabidopsis thaliana*

Gene	Locus	Cellular localization of the encoded protein	Enzymatic activity	Reference
OAS-A1	At4g14880	Cytosol	OASTL	(Barroso et al., 1995; Hell et al., 1994; Jost et al., 2000; Wirtz et al., 2004)
OAS-B	At2g43750	Chloroplasts	OASTL	(Hell et al., 1994; Hesse et al., 1999; Jost et al., 2000; Wirtz et al., 2004)
OAS-C	At3g59760	Mitochondria	OASTL	(Hesse et al., 1999; Jost et al., 2000; Wirtz et al., 2004)
CYS-D1	At3g04940	Cytosol	OASTL	(Hatzfeld et al., 2000; Yamaguchi et al., 2000)
CYS-D2	At5g28020	Cytosol	OASTL	(Hatzfeld et al., 2000; Yamaguchi et al., 2000)
CAS-C1	At3g61440	Mitochondria	CAS	(Garcia et al., 2010; Hatzfeld et al., 2000; Yamaguchi et al., 2000)
SCS	At3g03630	Chloroplasts	SSCS	(Bermudez et al., 2010)
DES1	At5g28030	Cytosol	DES	(Alvarez et al., 2010)

OASTL: O-acetylserine(thiol)lyase; CAS: β-cyanoalanine synthase; SSCS: S-sulfocysteine synthase; DES: L-cysteine desulfhydrase.

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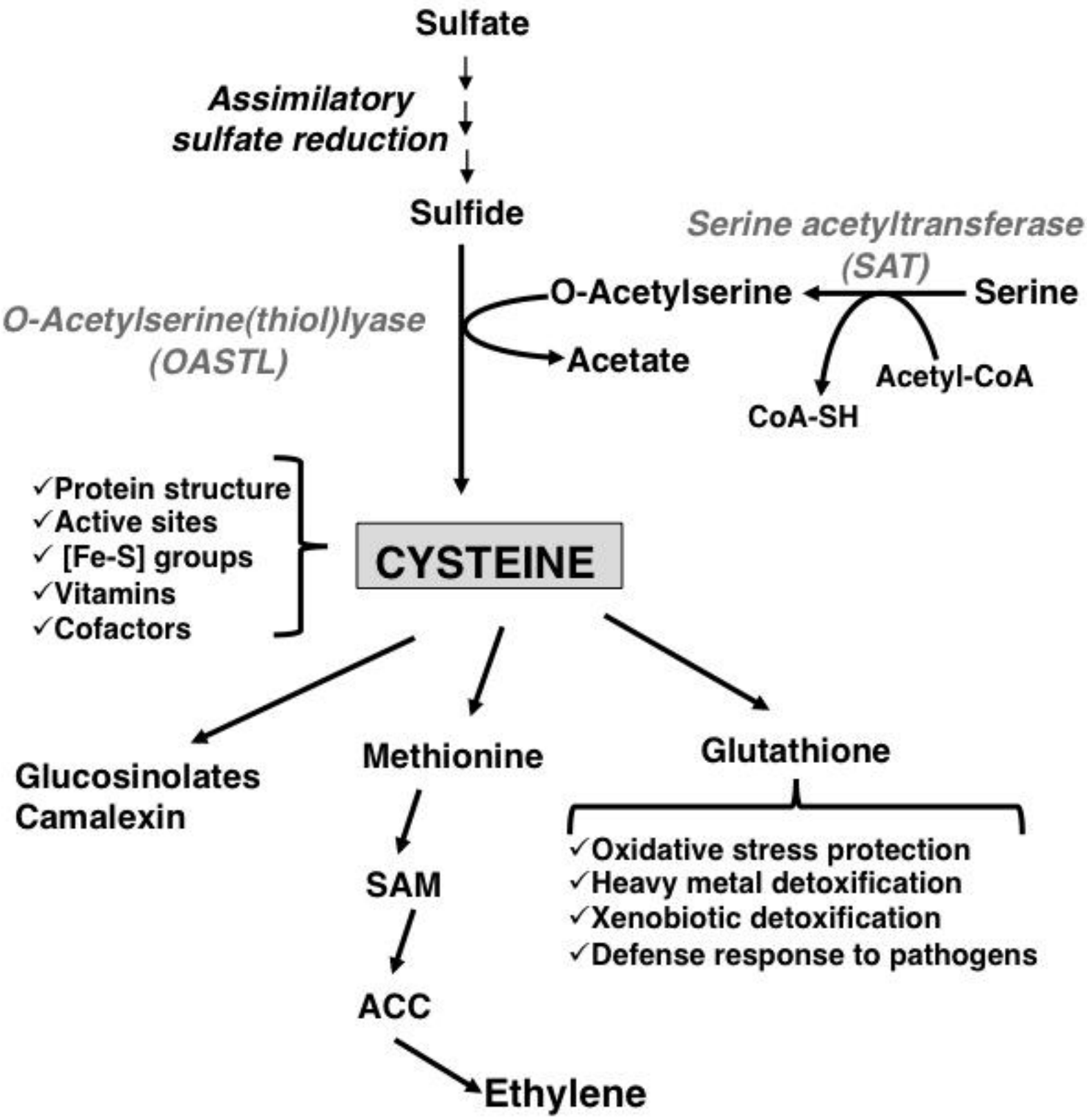

Figure 1. Enzymatic reactions involved in the biosynthesis of cysteine and overview of the roles of cysteine in plant metabolism

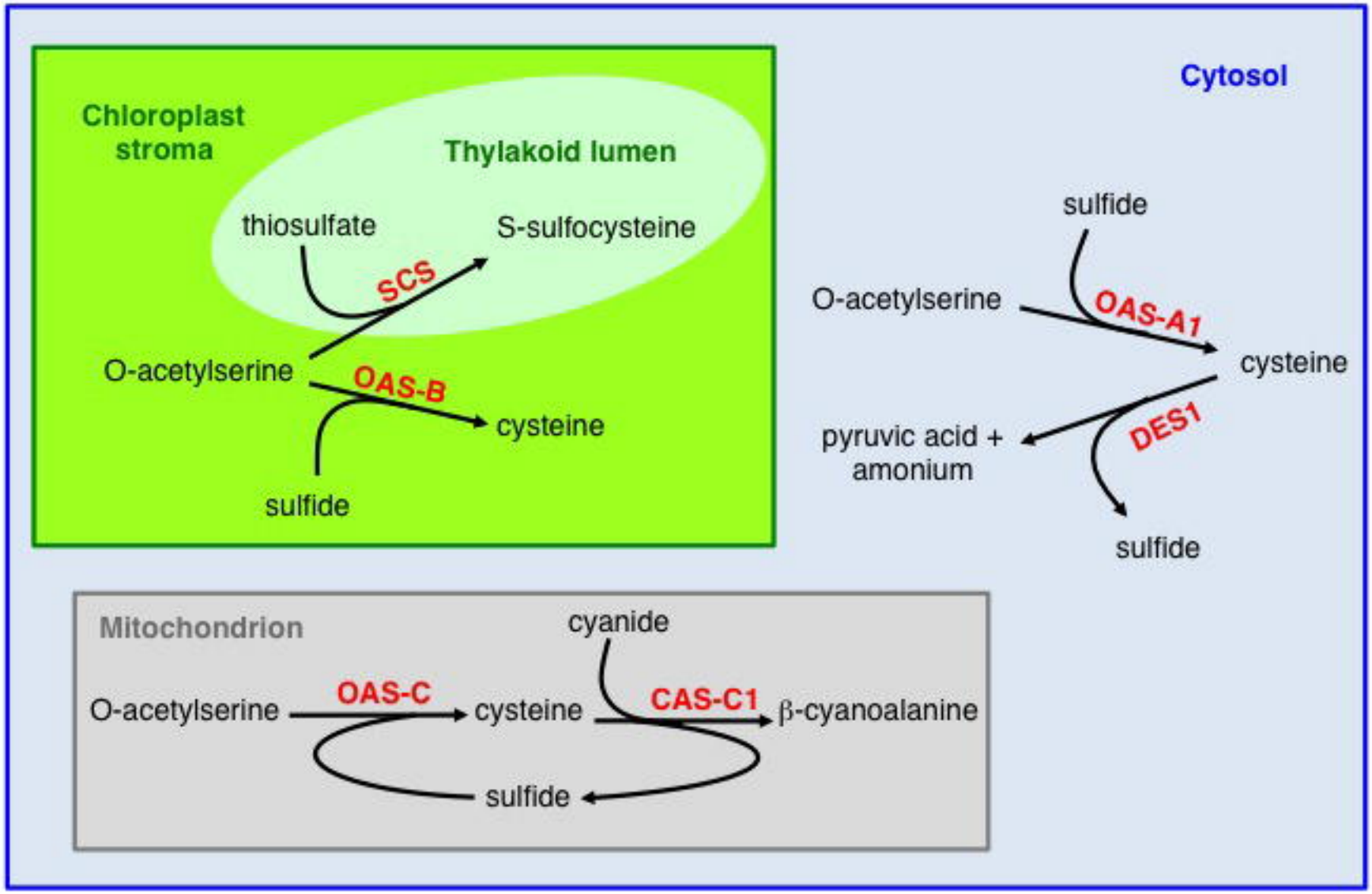

Figure 2. Subcellular localization of the different members of the OASTL enzyme family and reactions catalyzed by them in *A. thaliana*

Figure 3. Early-senescence phenotype of the *des1* mutant plants.
Wild-type Columbia-0 (Col-0) and *des1-1* mutant plants were grown side by side in soil for 4 weeks under long-day photoperiod (16h light/8h dark) and nutrient-sufficient conditions. A representative image is shown.

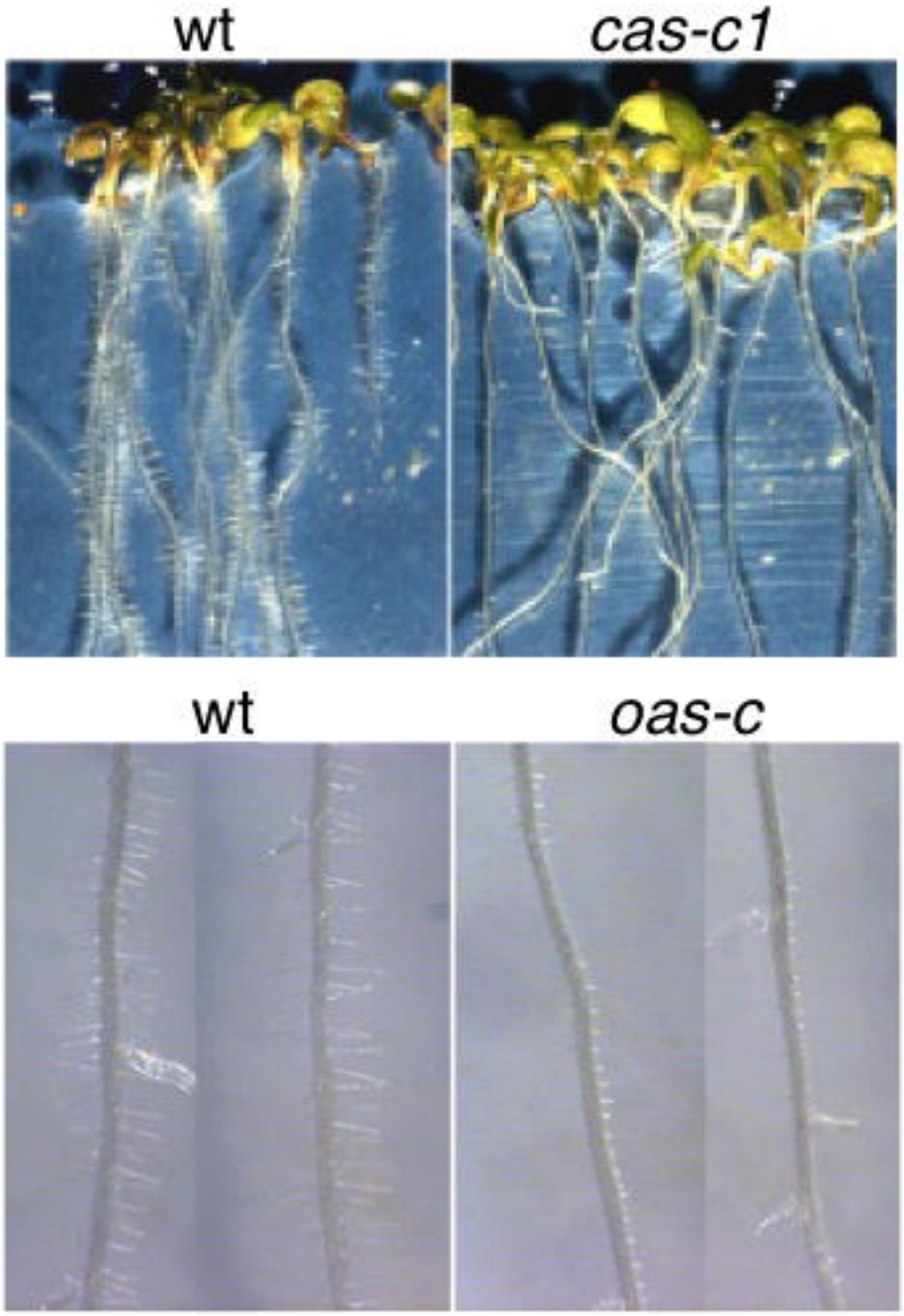

Figure 4. Root phenotype of the *cas-c1* and *oas-c* mutants.
Upper panel: Bright field images of 2-week-old wild type and *cas-c1* mutant seedlings, growing on vertical MS medium (Garcia et al., 2010) (www. plantcell.org; copyright American Society of Plant Biologists). Lower panel: Bright field images of roots from wild type and *oas-c* mutant seedlings, growing for 8 d on vertical MS medium (Alvarez et al., 2012c).

Figure 5. Phenotypes of the *scs* and *oas-b* mutants and the complemented *scs* line. Plants grown on soil under long-day (LD, 16h light/8h dark) and short-day (SD, 8h dark/ 16h light) conditions for 3 and 4 weeks, respectively. Details in (Bermudez et al., 2010) (www. plantcell.org; copyright American Society of Plant Biologists).

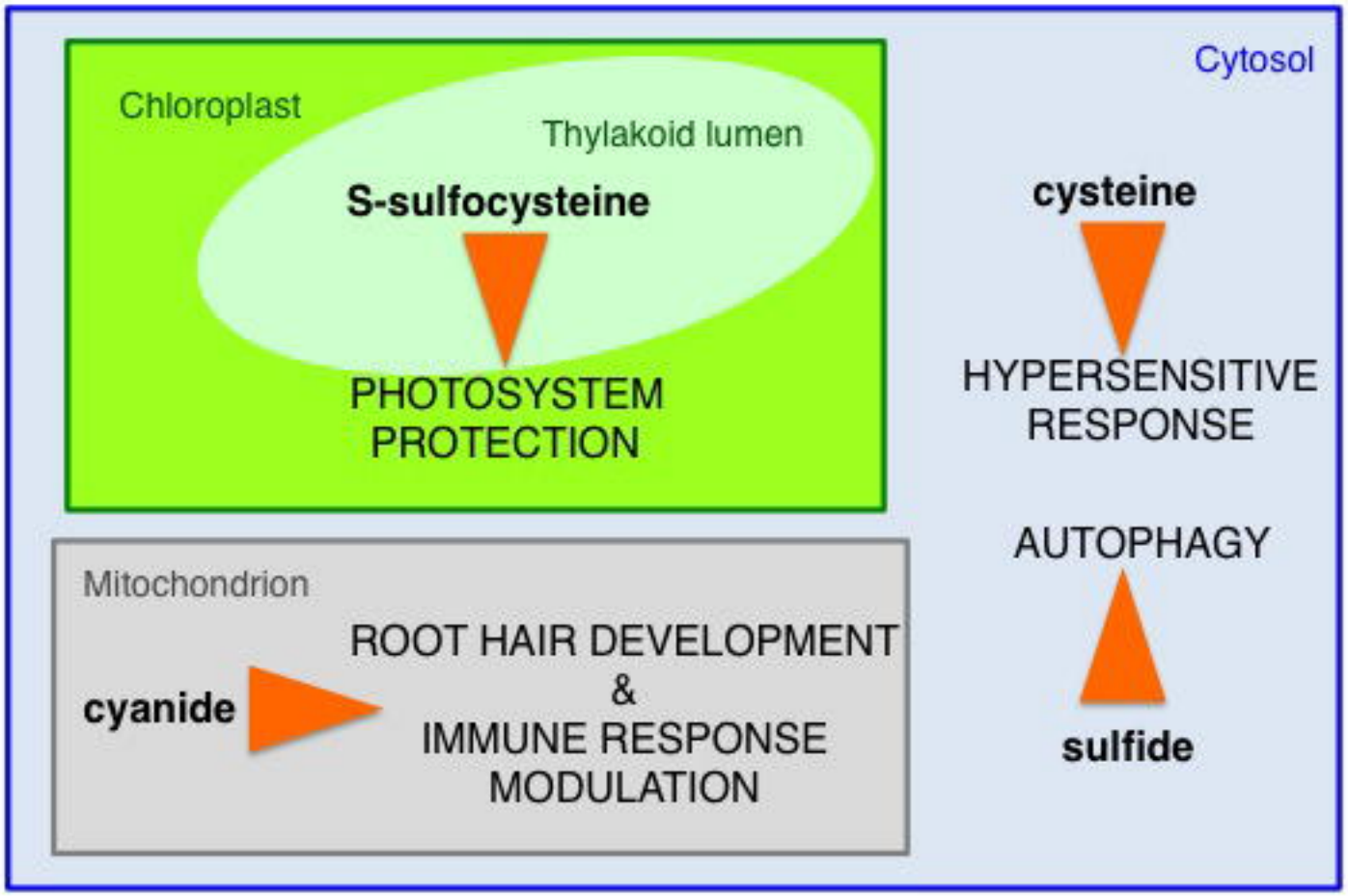

Figure 6. Processes regulated by cysteine and cysteine-related molecules in the *Arabidopsis* cell