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Something smells bad to plant pathogens: Production of hydrogen sulfide in plants and its role in plant defence responses

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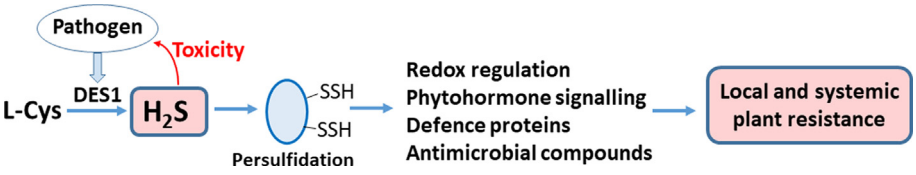
GRAPHICAL ABSTRACT



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ABSTRACT

Background: Sulfur and diverse sulfur-containing compounds constitute important components of plant defences against a wide array of microbial pathogens. Among them, hydrogen sulfide (H_2S) occupies a prominent position as a gaseous signalling molecule that plays multiple roles in regulation of plant growth, development and plant responses to stress conditions. Although the production of H_2S in plant cells has been discovered several decades ago, the underlying pathways of H_2S biosynthesis, metabolism and signalling were only recently uncovered.

Aim of the review: Here we review the current knowledge on the biosynthesis of H_2S in plant cells, with special attention to L-cysteine desulfhydrase (DES) as the key enzyme controlling H_2S levels biosynthesis in the cytosol of plant cells during plant growth, development and diverse abiotic and biotic stress conditions.

Key Scientific Concepts of Review: Recent advances have revealed molecular mechanisms of DES properties, functions and regulation involved in modulations of H_2S production during plant responses to abiotic and biotic stress stimuli. Studies on *des* mutants of the model plant *Arabidopsis thaliana* uncovered molecular mechanisms of H_2S action as a signalling and defence molecule in plant-pathogen interactions. Signalling pathways of H_2S include S-persulfidation of protein cysteines, a redox-based post-translational modification leading to activation of downstream components of H_2S signalling. Accumulated evidence shows DES and H2S implementation into salicylic acid signalling and activation of pathogenesis-related proteins and autophagy within plant immunity. Obtained knowledge on molecular mechanisms of H_2S action in plant defence responses opens new prospects in the search for crop varieties with increased resistance to bacterial and fungal pathogens.

Introduction

Hydrogen sulfide (H_2S) has been recognized as a multifaceted gasotransmitter involved in a diverse array of biological processes across all kingdoms including plants [1–3]. Since the first observation of H_2S emissions from leaves of several plant species by Wilson et al. 1978 [4], H_2S has emerged as a vital signalling

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molecule involved in the regulation of multiple mechanisms in plant growth and development and responses to external stimuli. H_2S was identified as an important component of signalling pathways regulating stomatal closure [5–7], root organogenesis [8] and photosynthesis [9]. Hydrogen sulfide also plays important roles in fruit biology and freshness and regulation of postharvest senescence of horticultural products. Application of H_2S in the form of aqueous solutions of sodium hydrosulfide (NaHS) or sodium sulfide (Na_2S) can decelerate fruit ripening and senescence in numerous fruits, partially by inhibiting the growth of fungal spores [10–11].

Under stress conditions, H_2S can shape plants ability of adaptation to diverse environmental stimuli by alleviating stress-induced injuries and activation of defence mechanisms [12–14]. Hydrogen sulfide can mediate enhancement of plant tolerance to salinity, drought, heavy metal and high-temperature stress, based on priming effect of H_2S on plant redox signalling, antioxidant capacity and specific components of cellular defence. [15–17]. Exogenous application of H_2S induces plant cross-adaptation to multiple abiotic stresses [18].

Signalling functions of H_2S in animal cells are known to be mediated namely by protein persulfidation of protein cysteines, interactions with metal centres in the protein active sites and reactions with S-nitrosothiols and electrophilic compounds [3]. Importantly, the biological functions of H_2S in plants involve interactions and cross-talk with signalling pathways of other plant gasotransmitters and reactive nitrogen and oxygen species [19–25]. Complex interactions of signalling pathways of H_2S and nitric oxide (NO) were revealed to regulate stomatal movement in plant leaves [19] where 8-mercapto-cGMP was shown as the active component of H_2S-mediated guard cell signalling [26]. Furthermore, biological functions of H_2S in plant growth, development and responses to abiotic stresses are determined by a dual role of H_2S in interactions with phytohormones. Endogenous H_2S levels are regulated by phytohormones, whereas H_2S can influence the production, transport, and signalling pathways of diverse plant hormones in plant physiological responses [27].

The role of H_2S in plant signalling and responses to abiotic stresses has attracted considerable attention and has been extensively reviewed elsewhere [14,18,21,28]. Within this special issue, Corpas and Palma [29] provide an excellent overview of the current state of knowledge on H_2S signalling in plants and potential application to increased plant performance under conditions of diverse environmental stresses.

It has become evident that similarly to reactive nitrogen and oxygen species, H_2S can perform a dual role in plant pathogenesis, i.e. signalling functions and direct inhibitory or toxic actions towards penetrating pathogens [30]. Crop fertilization with sulfur has been known for long to stimulate plant resistance, which is actually known to be mediated by H_2S. Pathogen resistance can be also potentiated by H_2S by induced expression of salicylic acid-dependent pathogen-related genes [30]. A substantial part of available published reports comprises rather descriptive studies performed by plant or fruit treatments with exogenous sulfide that has not provided deeper mechanistic insights into H_2S biological roles. L-cysteine desulfhydrase (DES1) has been shown to act as the key enzyme in the control of H_2S production and signalling in physiological conditions during plant growth and development as well as during plant-pathogen interactions. Moreover, H_2S has been recently reported to play a role in the regulation of plant autophagy, a key mechanism of plant innate immunity [31,32]. However, compared to H_2S role in plant responses to abiotic stresses, the sources, targets and mechanisms of H_2S action in diverse processes during plant-pathogen interactions are only partially uncovered. Major advances in this field have been achieved using *des1* mutant of *Arabidopsis thaliana* and need to be replicated and extended also in plant crop species and their relevant pathogens.

Up to our best knowledge, the actual state of the art in the field of H_2S functions in plant biotic interactions has not been previously reviewed. In this review, we focus namely to H_2S role in plant-pathogen interactions and we summarize in more detail the specific involvement of H_2S in plant responses to pathogen infection, with special attention dedicated to H_2S-producing enzyme L-cysteine desulfhydrase and its connections to plant sulfur metabolism.

L-cysteine and sulfide: A central role in plant sulfur metabolism and defence responses

In a central position within the plant primary metabolism and plant responses to stress conditions, amino acid L-cysteine (L-Cys) serves as a precursor of essential biomolecules and defence sulfur-containing metabolites [33,34]. L-Cys incorporated into peptide and protein molecules plays an outstanding role in redox-based signalling in various plant cell compartments. In a prominent place, the cysteine-containing tripeptide glutathione (GSH, γ–glutamyl-cysteinyl glycine) plays a crucial role in the maintenance of redox homeostasis and cellular protection to oxidative stress [35]. Protein cysteine thiols are targets of diverse post-translational modifications, which can strongly affect protein structure, activity, functions, and localization [36]. Oxidative modifications of thiol groups (-SH) in protein cysteines include a reversible formation of disulfides (-S-S-), sulfenic (-SOH) or sulfinic (-SO_2H) groups, whereas modifications caused by the action of signalling molecules NO and H_2S are represented by S-nitrosation and S-persulfidation, respectively [37–39]. Other examples of plant metabolites derived from L-Cys include amino acid methionine, enzyme cofactors like biotin and Fe-S clusters and S-adenosyl methionine, which provides methyl groups for methylation reactions in the biosynthesis of polyamines, phytosiderophores and phytohormone ethylene.

In the last decades, the involvement of diverse types of sulfur-containing compounds in plant defences and resistance to microbial pathogens has been widely uncovered. Besides the well-established role of GSH and GSH-dependent enzymes [40,41], L-Cys and its metabolites sulfide and carbonyl sulfide have been recognized within plant resistance mechanisms [42–44]. Sulfur-rich proteins, such as thionins, contribute to the disintegration of pathogen cell walls and induce the formation of ion channels in pathogen membranes [45,46]. Plant tissue challenge with microbial pathogens induces phytoalexins *de novo* [47–49]. Isocyanates as degradation products of glucosinolates represent another important group of antimicrobial compounds [50]. Elemental sulfur (S^0) is known to accumulate in vascular tissue upon fungal infections and to inhibit pathogen germination, respiration, and metabolism, possibly through interaction with protein thiol groups [51–53].

Collectively, a chemically diverse group of sulfur-containing metabolites, including elemental sulfur, glutathione, glucosinolates, phytoalexins and gaseous H_2S are involved in pathogen resistance. Their occurrence is species-specific and in a large extent influenced by the sulphur nutritional status of the plant.

Synthesis and catabolism of L-cysteine as H_2S precursor in the plant cytosol

L-Cysteine, as a potential donor molecule of reduced sulfur, is produced in the last step of sulfate assimilation in plants by incorporation of sulfide into O-acetylserine catalysed by O-acetylserine (thiol)lyase (OAS-TL), which is found in various isoforms in the cytosol, mitochondria and chloroplasts [33,54–56]. The cytosol of

plant cells is thus the main cellular compartment of L-Cys biosynthesis. As a result of very high activity of cytosolic OAS-TL isoform OAS-A1, usual cytosolic L-Cys concentrations range around 300 μM, whereas in other compartments L-Cys is found at levels lower than 10 μM [57]. Due to its high reactivity, increasing concentrations of L-Cys potentially cause toxic effects to plant cells. L-Cys is an effective reductant of iron (III) to iron (II) ions, which participate in Fenton-type reactions with reactive oxygen species (ROS) causing oxidative damage of cellular components [58,59]. For this reason, the maintenance of L-Cys homeostasis by the coordinate action of key enzymes of its biosynthesis and catabolism, i.e. OAS-A1 and L-cysteine desulfhydrase (DES), is of utmost importance for the proper functioning of plant metabolism under physiological and stress conditions.

The most abundant cytosolic OAS-TL isoform OAS-A1 is involved in plant responses to abiotic stress, namely to heavy metals exposure, through metal-chelating activity of phytochelatins, synthesized from L-Cys with GSH as an intermediate [35]. A major contribution to understanding the role of the key enzymes in L-Cys biosynthesis and catabolism was provided by studies of Arabidopsis *oas-a1* and *des1* mutants, respectively [33,60,61]. The knockout *oas-a1.1* and *oas-a1.2* mutants were characterized by decreased intracellular L-Cys and glutathione levels. Compromised antioxidant capacity results in perturbation of H_2O_2 homeostasis, as documented by spontaneous cell death lesions occurring in leaves of *oas* mutants [60]. Mutation of the *DES1* gene results in elevated total Cys content caused by reduced total Cys desulfuration activity in leaves. Arabidopsis *des1* mutants show premature leaf senescence, whereas enhanced antioxidant defences and tolerance to oxidative stress [61].

A 25% decrease of L-Cys concentration was observed in the *oas-a1* mutant, in contrast to a 25% increase of L-Cys levels in a *des1* mutant which did not cause any toxic effects to the mutant plants. *OAS-A1* down-regulation resulted in higher ROS levels, likely associated with defective homeostasis of H_2S, whereas the DES1 deficit was accompanied with decreased ROS. In consequence, decreased GSH levels in OAS-A1 deficient plants were associated with an increased ratio of oxidized glutathione. Subsequently, *oas-a1* mutants showed compromised tolerance to cadmium exposure, in comparison to wild-type plants. The overall oxidative stress induced by OAS-A1 deficiency was evident even under control growth conditions when plants showed the spontaneous formation of leaf cell death lesions [33,60,61].

The chloroplastic OAS-TL isoform in *Arabidopsis thaliana* has been described as an S-sulfocysteine synthase (SSCS) enzyme which has a crucial role in the proper photosynthetic performance of the chloroplast under long-day growth conditions. SSCS is located in the thylakoid lumen and it was suggested to function as a sensor to detect accumulated thiosulfate caused by ineffective removal of ROS under conditions of excess light [62–64].

Together with plant OAS-TL, mitochondrial β-cyanoalanine synthases (CAS-C1) belong to the large superfamily of pyridoxal 5′-phosphate-dependent enzymes together with OAS-TL. As a mechanism of cyanide detoxification, CAS catalyses the biosynthesis of the nonprotein amino acid β-cyano-Ala from L-Cys and cyanide, producing sulfide as a product [65,66]; however, the contribution of CAS to H_2S production is highly variable among plant species. Nine CAS genomic sequences were reported in *A. thaliana* [67]. Using T-DNA insertion mutants, cytosolic Bsas1.1, plastidic Bsas2.1, and mitochondrial Bsas2.2 were found to play important roles in L-Cys biosynthesis, with a major contribution of cytosolic Bsas1.1 in leaves and root, and mitochondrial Bsas2.2 in the root.

Addition of O-acetylserine inhibits emissions of gaseous H_2S from plant tissues and increased L-Cys levels [68,69]. On the other hand, compounds known to inhibit GSH biosynthesis induce H_2S emission, suggesting that under conditions when biosynthetic pathways consuming L-Cys are inhibited, sulfides are emitted in the form of gaseous H_2S [70]. A correlation between enzymatically-produced H_2S and the total amount of sulfur was observed in *Brassica napus* [71]. With increased sulfur content, DES1 activity was observed to decrease whereas OAS-TL activity decreased. However, later reports found that under sulfur deficiency, plants showed up-regulation of both OAS-TL and DES1 [72].

The schematic overview of known biochemical pathways of H_2S biosynthesis and conversion in plant cells highlight the central role of DES1 in cytosolic H_2S productions (Fig. 1). Other enzymes such as CAS-C1, D-cysteine desulfhydrase (β-DCD), L-cysteine desulfurase (DSF) and ferredoxin are capable to contribute to H_2S production in other plant cell compartments, but to which extent this might occur in different plant species under specific growth or stress conditions is largely unknown. Current knowledge thus demonstrates that L-Cys occupies a central position in plant sulphur metabolism as a reduced sulfur donor in the biosynthesis of defence compounds. Besides the involvement of L-Cys and its metabolites in redox signalling within plant cell compartments, cytosolic and mitochondrial L-Cys play crucial roles in plant immunity and cyanide detoxification, respectively. The functions of L-Cys in plant responses to pathogen challenge are in a large extent mediated by L-Cys-derived H_2S, as discussed in the next sections of this review.

L-cysteine desulfhydrase: A key enzyme of H_2S production in plants

L-Cysteine degradation in plant cytosol is known to proceed in a reaction catalysed by L-cysteine desulfhydrase (DES), leading to H_2S, pyruvate and ammonia. Cytosolic levels of L-Cys and also of H_2S are therefore controlled by activities of OAS-TL and DES [72,73]. Detailed characterization of *des1* mutants of *A. thaliana*, together with pharmacological approaches using DES1 inhibitors as well as H_2S donors and scavengers, have recently provided valuable insights into the role of DES1 and H_2S in signalling pathways of plant responses to biotic and abiotic stress stimuli [44,74–76].

L-cysteine desulfhydrase (DES1, EC 4.4.1.1.)) is the main enzyme of L-Cys catabolism in plant cytosol, which catalyses L-Cys decomposition to pyruvate, ammonia and H_2S in a stoichiometric ratio 1:1:1. DES1 regulates cytosolic L-Cys levels together with major cytosolic OAS-TL, highly active OAS-A1 involved in L-Cys biosynthesis [33,54]. Plant DES1 belongs to the family of O-acetylserin (thiol)lyases (OAS-TL), which in *A. thaliana* comprises 8 described members involved in sulfur metabolism (Table 1).

This enzyme was described for the first time based on sequence homology to other members of OAS-TL family during Arabidopsis genome sequencing. Originally, the enzyme was termed as CS-LIKE according to its cysteine synthase-like activity. Alvarez et al. [61] achieved production and purification of recombinant Arabidopsis DES1 in *E. coli* and performed detailed in vitro characterization of enzyme molecular properties. Similarly to other members of OAS-TL family, DES1 requires pyridoxal phosphate (PLP) as a cofactor and contains all conserved amino acid residues involved in PLP binding. On the other hand, DES1 differs from other members of OAS-TL family in the sequence of β8A-β9A loop, which is otherwise highly conserved in OAS-TLs due to its role in protein interaction with serine O-acetyltransferases [55,77].

Purification of recombinant DES1 by affinity chromatography results in increased cysteine desulfhydrase activity of the protein but decreased O-acetylserine lyase activity, in agreement with enzyme primary role in cysteine degradation. This is also supported by enzyme higher activity towards L-Cys, measured by the value of Michaelis constant K_M, which is 14-times lower for L-Cys in DES reaction compared to K_M value for O-acetylserine in OAS-TL reaction. However, the limit reaction rate is quite low, determined as 0.04 $\mu mol.min^{-1}.mg^{-1}$ for purified Arabidopsis

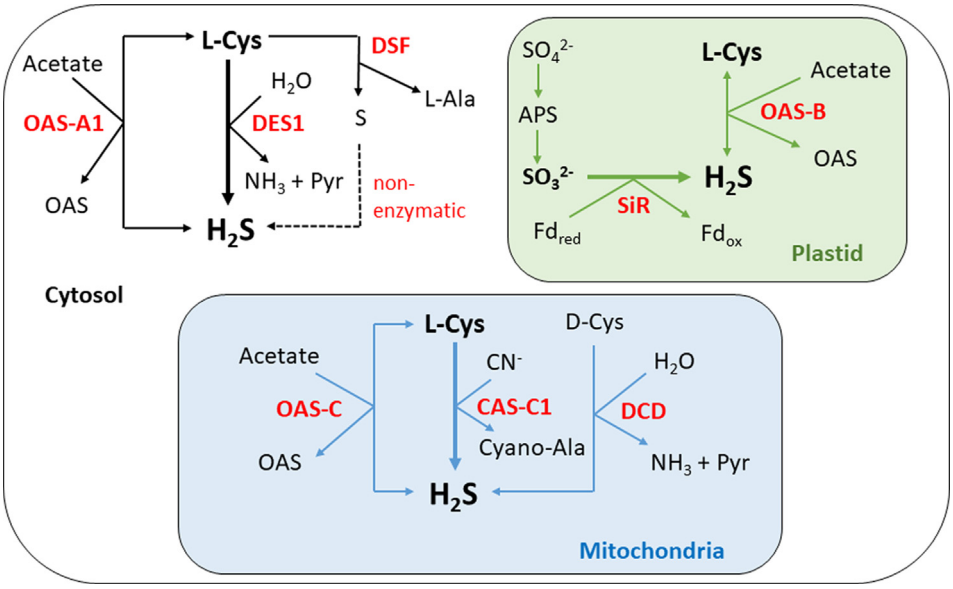

Fig. 1. Overview of H_2S production in plant cells. APS, adenosine phosphosulfate; CAS-C1, β-cyanoalanine synthase (EC 4.4.1.9); Cyano-Ala, cyanoalanine; DCD, D-cysteine desulfhydrase (EC 4.4.1.15.); DES1, L-cysteine desulfhydrase (EC 4.4.1.2.); DSF, L-cysteine desulfurase (EC 2.8.1.7); Fd, ferredoxin; OAS, O-acetylserine; OAS-A1, cytosolic O-acetylserine (thiol)lyase; OAS-B, plastidial O-acetylserine (thiol)lyase; OAS-C, mitochondrial O-acetylserine (thiol)lyase; Pyr, pyruvate; SiR, sulfite reductase (EC 1.8.7.1.).

Table 1
Overview of the OAS-TL gene family in *A. thaliana*. CAS, β–cyanoalanine synthase; DES, L–cysteine desulfhydrase; OAS-TL, *O*–acetylserine (thiol)lyase; SSCS, *S*–sulfocysteine synthase.

Gene	Locus	Localization	Enzyme activity
OAS-A1	At4g14880	Cytosol	OAS-TL
OAS-B	At2g43750	Chloroplasts	OAS-TL
OAS-C	At3g59760	Mitochondria	OAS-TL
CYS-D1	At3g04940	Cytosol	OAS-TL
CYS-D2	At5g28020	Cytosol	OAS-TL
CAS-C1	At3g61440	Mitochondria	CAS
SCS	At3g03630	Chloroplasts	SSCS
DES1	At5g28030	Cytosol	DES

DES1. Purified AtDES1 also possesses D-cysteine desulfhydrase activity, but one order of magnitude lower compared to L-Cys desulfhydrase activity [61].

A homologous *DES1* gene was isolated from rapeseed (*Brassica napus*) and sequenced, showing 85% homology to Arabidopsis gene and coding a 323 amino acid polypeptide of 34.5 kDa molecular weight [78]. BnDES1 was found highly expressed in rapeseed flowers, whereas the expression levels in vegetative tissues were much lower. Similarly to AtDES1, BnDES12 also shows a minor O-acetylserine (thiol)lyase activity. A recent report revealed OAS-TL family in *Solanum lycopersicum*, where measurable DES activity was found in some isoforms, namely SlOAS4 and SlOAS6 [79]. Reported pH optimum values for plant DES range from 8.0 in tobacco [80], 9.0 in Brassica [78] to 10.0 in *A. thaliana* [81]; however, the physiological relevance of these differences in DES pH optima is not known. Based on the published reports, DES1 enzyme catalysing L-Cys desulfhydration has been established as the main sources of the endogenous production of H_2S in the plant cytosol, at least in *A. thaliana*. Moreover, it has been shown that DES1 is regulated by plant hormones on the transcriptional level, enabling DES1 and H_2S level regulation in response to stress stimuli.

L-cysteine desulfhydrase activity has not been detected in animals, where H_2S production has been demonstrated to be catalysed by cystathionine–γ–lyase and cystathionine-β-synthase [82] or 3-mercaptopyruvate sulfur transferase [83]. However, L-cysteine desulfhydrase was discovered to operate in some bacteria, e.g. *Escherichia coli* [84,85].

It should be noted that D-cysteine desulfhydrase (EC 4.4.1.15), which catalyses the conversion of D-cysteine to the same products as DES1, including H_2S, represent a completely different enzyme both in protein structure and biochemical properties [86]. D-cysteine desulfhydrase activity has been observed in multiple plant species including Arabidopsis, where two genes At3g26115 and At1g48420 coding proteins with D-cysteine desulfhydrase activity were identified [87,88]. Interestingly, the biological function of D-cysteine as well as of D-enantiomers of other amino acid is still not known. One of the suggested functions of D-cysteine desulfhydrase might be degradation of malformin, a phytotoxic peptide produced by *Aspergillus niger* containing D-cysteine [89].

Production of H_2S can be catalysed also by cytosolic L-cysteine desulfurase (EC 2.8.1.7) when using L-cysteine methyl ester as a substrate [90]. The main role of L-cysteine desulfurases is to catalyse L-Cys desulfuration to give L-alanine and elemental sulfur. Proteins showing L-cysteine desulfurase have been identified in the cytosol, chloroplast and mitochondria, where it provides elemental sulfur for the biosynthesis of biotin, thiamine and Fe-S clusters [91,92].

DES1 regulates H_2S production and signalling during plant growth and abiotic stresses

As already mentioned, DES1 is the specific source for the production of cytosolic H_2S, involved in signalling pathways of vital plant processes like autophagy and stomatal regulation. During the development of Arabidopsis plants, the highest *DES1* gene expression was found in leaves of 14-days old seedlings and 35-old plants just after the termination of flowering, whereas the lowest expression levels were observed in rosettes of 20-days old plants before the appearance of visible buds [93]. At the tissue level, *DES1* transcripts were abundant in mesophyll and epidermal cells, including guard cells, in cells surrounding hydathode pores, in trichomes and flowers.

It is known that mutations in AtDES1 induce leaf senescence, accompanied by higher expression of genes involved in plant ageing and increase levels of related transcription factors. Absence of

DES1 activity leads to substantially decreased overall desulfurase activity in leaves, associated with increased L-Cys levels [61]. Furthermore, DES1 deficiency in Arabidopsis results in an accumulation of various isoforms of autophagy-related proteins 8 (ATG8), as a sign of activated autophagy processes [31]. The DES1 reaction product, H_2S, is likely involved in autophagy regulation, as suggested by the inhibitory effect of exogenous H_2S to the accumulation of ATG8 proteins in *des1* mutants.

Metabolic rates of L-Cys and H_2S production are closely related to the plant nitrogen uptake. Plants under high nitrogen nutrition conditions contain higher activities of OAS-TL and DES1, increased level of sulfur-containing compounds and decreased sulfate levels compared to plants grown in nitrogen-deficient conditions. These findings suggest that sufficient nitrogen uptake enables a higher rate of sulfur incorporation into proteins. The observed higher DES1 activity can serve as a protective factor to avoid excessive L-Cys accumulation [72].

Interestingly, decreased levels of nitric oxide (NO) were observed in *des1* mutant, suggesting DES1 is involved by an unknown mechanism in production this gaseous signalling compound in stomata [94]. Currently, it has been recognized that H_2S regulates multiple developmental processes and stress responses in interaction with signalling pathways plant hormones [27] including signalling gasotransmitters NO [95] and carbon monoxide [96] or reactive oxygen species like hydrogen peroxide [28].

The role of H_2S in stomata closure has been extensively studied [5–7,94]. DES1 is involved in the signalling pathway of abscisic acid (ABA) in the leaf stomata, where it participates in the regulation of guard cell movements in stomata closure and opening. ABA is known to induce *DES1* expression in wild-type plants, whereas in the ABA-nonresponsive *des1* mutant stomata closure can be induced by exogenous H_2S [97].

Treatment with H_2S, provided as an aqueous solution of NaHS, promotes lateral roots in tomato seedlings with increased auxin levels, suggesting H_2S produced by DES1 is involved in the auxin signalling pathway regulating lateral roots formation [98]. A role for DES1 has been proposed in phytohormone-induced programmed cell death in the aleurone layer of wheat [99]. This was evidenced by observations that gibberellins cause decreased DES activity in wheat aleurone and that H_2S could prevent the gibberellin-triggered programmed cell death of aleurone cells.

Expression and activity of DES1 in plants were revealed to be modulated by diverse external conditions and stress stimuli [33]. The function of DES1 in response to abiotic stress-mediated by H_2S production has been reported in increased tolerance to drought [100], osmotic stress [95] and heat stress [96]. Conversely, *des1* mutants showed increased resistance to cadmium exposure, likely mediated by increased levels of L-Cys used to synthesize cadmium-chelating phytochelatins [33,61]. DES1 transcript levels and activity are induced in Arabidopsis by cold and salinity stress, hydrogen peroxide, and ABA [75]. Treatment of maize seedlings by salicylic acid induces DES1 activity, leading to H_2S accumulation involved in increased tolerance to high temperatures [101]. SA-induced tolerance to high temperatures was found potentiated by H_2S, although it did not affect SA levels or enzymes of SA biosynthesis. The role of H_2S in plant tolerance to dehydration stress was demonstrated with H_2S-mediated activation of carbonic anhydrase and OAS-TL activity, whereas both dehydration stress and an exogenous application of NaHS induced DES1 activity increasing plant H_2S levels produced from accumulated Cys [102]. In salt-stressed tobacco, high NaCl treatment stimulated CAS and CS activities leading to in H_2S accumulation in tobacco leaves, whereas sulphite reductase activity was decreased [103].It has been recognized that the complex biological connections between H_2S and other phytohormones and plant regulators include diverse pathways depending on the plant species and tissue. The observed cross-talk of H_2S and plant hormones suggests that H_2S can serve as an integral molecule of plant hormone signalling. H_2S is known to control the expression of genes involved in phytohormone biosynthesis, which might alter actual proportions of hormone levels controlling multiple processes during plant growth and stress responses [27]. Furthermore, similarly to reactive nitrogen and oxygen species, H_2S-dependent post-translational modification of proteins and enzyme such as cysteine persulfidation can affect the distribution and signalling of plant hormones. Current evidence in suggest that NO and H_2S act in plants synergistically or antagonistically, depending on their actual levels, as signalling compounds or damage effectors. An important part of their signalling effects proceeds via reversible redox-based modifications of protein cysteines, which include S-nitrosation and persulfidation for NO and H_2S, respectively [23].

Taken together, results of experimental studies indicate that DES1 and its product H_2S contribute to the establishment of plant abiotic stress tolerance, likely mediated by regulation of stress gene transcription, metabolism of reactive oxygen species and auxin signalling pathways.

DES1-mediated H_2S production in plant defences

Current accumulated knowledge support a functional implementation of DES1 into signalling pathways of salicylic acid (SA), the key plant hormones in responses to microbial pathogens as well as in plant growth and development [104,105]. WRKY transcription factors are key regulators of specific plant developmental processes, including seed dormancy, seed germination, and senescence and also plant responses to biotic and abiotic stresses [106]. Expression levels of multiple WRKY members were previously found to be modulated by pathogen infection or SA treatment [107] and WRKY transcription factors are known to down-regulate the expression of DES in *A. thaliana* [108]. Transcript levels of *WRKY54*, which serves as a transcription factor regulating gene expression of PR proteins were increased in *des1* mutants and decreased in *oas-a1* mutants [60]. Simultaneously, *des1* mutants showed a lower degree of L-glutathione oxidation compared to *oas-a1* mutants. Higher levels of L-Cys in *des1* mutants, resulting in lower intracellular redox potential, thus can contribute to increased plant resistance to invading pathogens. This has been further confirmed in a subsequent study of Alvarez et al. [42], which characterized Arabidopsis *des1* mutants as more resistant to both biotrophic and necrotrophic pathogens, whereas *oas-a1* mutants showed compromised pathogen resistance. In parallel, higher levels of SA, putatively involved in long-distance plant signalling, were observed in *des1* mutants. Transcriptomic analysis showed induction of four PR proteins including defensins PDF1-2a and PDF1-2b. Collectively, high Cys-associated decreased intracellular redox potential might play an important role within plant defence to pathogens; however, this mechanism requires further experimental investigations in Arabidopsis and other plant species.

Arabidopsis *cad2-1* mutants show approx. 70% decreased levels of L-glutathione but unchanged levels of L-Cys, as compared to wild-type plants, unlike *oas-a1* mutants where both L-Cys and glutathione levels are reduced. As the repression of WRKY54 was not found in *cad2-1* mutants, suggesting the inhibition of PR expression in *oas-a1* mutants was caused by decreased L-Cys levels. Interestingly, members of the WRKY family were recently identified to pose binding capacity to the promotor of DES1 gene and regulate its expression [108]. Furthermore, *oas-a1* mutant plants lack the capacity of the hypersensitive reaction, which can be restored by addition of L-Cys but not glutathione, in agreement with a specific requirement for L-Cys in incompatible interactions in plant pathogenesis [33,42]. The involvement of L-Cys in plant immunity was tested on *oas-a1* plants exposed to a virulent bacterial pathogen

Pseudomonas syringae pv. *tomato* DC3000, which produces effectors suppressing plant immunity induced by pathogen molecular patterns. Arabidopsis *oas-a1* mutant plants showed increased susceptibility to this pathogen [109].

Development of Arabidopsis mutants in two genes of cysteine desulfhydrases enabled to study the function of DES1 and H_2S in plant tolerance biotic and abiotic stress stimuli [75]. Transcript levels of DES1 and D-cysteine desulfhydrase (EC 4.4.1.15) were increased by bacterial pathogen as well as diverse abiotic stress conditions including cold, dehydration, salt and hydrogen peroxide treatment; however, with different timing. The highest increase in DES1 expression and activity was detected from 1 to 3 h after pathogen infection, whereas increased DES1 mRNA continued several hours after the abiotic stress treatment. Compared to wild-type plants, DES1 overexpressors showed lower counts of bacterial cells in infected tissues, in contrast to increased counts of pathogen cells in *DES1* knock-outs. Plant defence responses were induced and suppressed by NaHS or H_2S scavenger hypotaurine, respectively. Moreover, expression of PR protein genes was induced in DES overexpressors by NaHS treatment, while it was found increased in *DES1* knock-down mutants and by H_2S scavenger. Collectively, these data support the functional role of DES1 enzyme in H_2S production involved in the regulation of plant resistance mechanisms, putatively mediated by activation of salicylic acid-dependent signalling and defence genes [75].

Interestingly, plants under sulfur deficient conditions are able to uptake gaseous H_2S or carbonyl sulfide (OCS) from the environment, whereas H_2S and OCS are released on pathogen infection, resulting in an overall decrease of sulfur content in infected plants [110]. During the flowering period, plant responses to infection were slower with decreased and decelerated emission of H_2S and OCS, suggesting differential regulation of sulfur metabolism in the vegetative and reproductive stage of plant development. Moreover, emitted gaseous molecules can serve as long-distance signals to alert closely located plants about ongoing infection in their proximity. In agreement with this hypothesis, increased amount of thiol-containing compounds was detected in plant growing close to infected plants, compared to control plants grown separately [111].

In summary, the use of transgenic plants with modulated DES1 activity and endogenous H_2S level, in combination with exogenous treatments with H_2S donors or scavengers, confirm proposed protective role of H_2S in biotic stress resistance.

Mechanisms of H_2S action in plant-pathogen interactions

As evident, major advances in the detailed understanding of H_2S sources and functions in plant metabolism and stress responses have been obtained in the model plant *A. thaliana*, with available mutants of key enzymes of sulphur metabolism. In contrast, the understanding of the role and mechanisms of H_2S action in defence responses of crop plants is quite limited (Table 2).

Early studies found killing grape mildews by H_2S fumigation in a close jar [112] and completely inhibited germination of *Botrytis cinerea* spores sowed in a saturated solution of H_2S [113]. This was confirmed by a more extensive study on a set of fungal phytopathogens (including *B. cinerea, Cladosporium herbarum, Fusicladium dendriticum, Monilia cinerea and M. fructigena, Pencillium verdicatum, Physalospora miyabeana*) which showed H_2S acting as a general poison toxic at a low concentration to all used fungi [114]. Importantly, this study noted the conversion of sulphur to volatile H_2S, which mediates the toxic effects previously attributed to the sulphur treatments.

The enzyme DES1 was identified as the H_2S source in rapeseed plants infected by a fungal pathogen *Pyrenopeziza brassicae*, which resulted in a 50% increase of DES1 activity [72]. Fungal infection of the grapevine by grape powdery mildew (*Uncinula necator*) induced an increased release of H_2S, namely during the early phase of the infection; however, it strongly decreased 10 days after infection [43]. An application of elemental sulfur to powdery mildew-infected grape leaves showed the highest efficiency when applied in the early phase of pathogenesis prior to the formation of fungal appressoria in penetrated leaf cells [115]. It was estimated that uptake of 10 μM/h of H_2S by the pathogen would provide a fungicidal effect. Role of H_2S in plant defence against fungal pathogen was evidenced by significant increase of H_2S emissions from crops challenged by fungal infection [72,111]. Collectively, in a similar manner to model plants, crops have been demonstrated to exert capabilities to respond to fungal infection by modulations of L-Cys metabolism, H_2S emissions and increased levels of GSH and phytoalexins [116].

Recently, it was found that H_2S could extend postharvest storage of fresh-cut pears and inhibit the growth of fungal pathogens *Aspergillus niger* and *Penicillium expansum* [117]. The inhibitory effects of H_2S to these fungal pathogens both on inoculated fruits and in vitro culture was confirmed for several fruits including apple, lemon, kiwi and tomato [118]. Fumigation with H_2S released from NaHS solution inhibited the growth of fungal pathogens *Rhizopus nigricans*, *Mucor rouxianus* and *Geotrichum candidum* on slices of sweet potato (*Ipomoea batatas*); however, the molecular mechanism of the antifungal H_2S action has not been elucidated [119]. Similarly, strawberry (*Fragaria ananassa*) fruits treated with NaHS solution, or with a combination of NaHS and a NO donor, resulted in increased activities of potentially antifungal enzymes chitinase and beta-1,3-glucanase; however, if this effect can contribute to fruit resistance to fungal contamination and decay has not been tested [120].

Recently, the antimicrobial effect of H_2S was corroborated also for a microbial pathogen using Arabidopsis plants infected with *P. syringae* pv. *tomato* DC3000 [75]. Plants overexpressing LCD and DCD1 had lower bacterial counts compared to WT plants, unlike LCD and DCD1 knockdown plants exhibiting higher bacterial infection. Furthermore, both LCD and DCD1 overexpressors and plants treated with an NaHS solution showed higher levels of transcription of pathogenesis-related genes. In vitro, *P. syringae* pv. *phaseolicola* were found to be resistant to low levels of H_2S, whereas high doses of NaHS, Na_2S and a mitochondria-targeted H_2S donor AP39 inhibited cell growth, which was mediated by excision of a genomic island from the bacterial genome [121]. It has been suggested that H_2S emitted from the plants in response to bacterial challenges can modify the genomic structure of invading bacteria and thus affect their virulence, which might be exploited to increase crops resistance.

It would be not surprising to found that pathogens have evolved mechanisms for efficient H_2S removal and detoxification. H_2S is known to block cell respiration as a strong inhibitor of cytochrome *c* oxidase [122], so its elimination is vital to enable the growth of the microbial pathogen in a low oxygen environment, as in bacterial biofilms, plant xylem vessels or root tissues. In plant pathogens *Xylella fastidiosa* and *Agrobacterium tumefaciens*, the biofilm growth-associated repressor (BigR) regulates transcription of the bigR operon, which is important for H_2S detoxification through the action of a sulfur dioxygenase in conjunction with a sulfite exporter [123]. It was shown that the respiratory oxidase cytochrome bd in the model microorganism *Escherichia coli* is resistant to H_2S inhibition [124]; however, it seems that this mechanism of respiration resistance to H_2S inhibition is present only in enterobacteria.

Hydrogen sulfide in biological systems occurs as diprotonated gaseous H_2S as well as HS^- anion, which co-exist in a chemical equilibrium [1,37,94]. Gaseous H_2S can diffuse freely across the cell membranes and migrate outside of the plant tissues, which will

Table 2
Summary of published studies on the role of H_2S in plant defences to pathogens.

Plant species	Pathogen	Treatment	Observed effects	Source
Vitis vinifera (grapes)	*Uncinula necator*	Elemental sulfur	Fungicidal effects of sulfur-derived H_2S	[112]
n.a.	*Botrytis cinerea*	Saturated solution of H_2S	Inhibition of spore germination	[113]
n.a.	*Botrytis cinerea, Cladosporium herbarumFusicladium dendriticumMonilia cinereaMonilia fructigena Pencillium verdicatum Physalospora miyabeana*	Fumigation with H_2S	Fungicidal effect	[114]
Brassica napus	*Pyrenopeziza brassicae*	*n.a.*	Increased DES1 activity	[72]
Vitis vinifera	*Uncinula necator*	*n.a.*	Increased H_2S release in the early phase of infection	[43]
Vitis vinifera (leaves)	*Uncinula necator*	Elemental sulfur applied in the early phase of pathogenesis	Uptake of 10 μM/h of H_2S by the pathogen provides fungicidal effect	[115]
Brassica napus	*Sclerotinia sclerotiorum*	*n.a.*	Increased H_2S release	[111]
Actinidia deliciosaCitrus sinensisCitrus reticulataMalus domesticaPyrus bretschneideri Solanum lycopersicum	*Aspergillus nigerPenicillium italicum*	Fumigation with H_2S released from NaHS solution	Reduced postharvest decay of fruits induced by fungal pathogensInhibition of spore germination, germ tube elongation and mycelial growth	[118]
Ipomoea batatas	*Rhizopus nigricans*, *Mucor rouxianus*, *Geotrichum candidum*	Fumigation with H_2S released from NaHS solution	Inhibition of fungal growth	[119]
Pyrus pyrifolia	*Aspergillus niger, Penicillium expansum*	H_2S fumigation	Inhibition of fungal growth	[117]
Fragaria ananassa (strawberry)	n.a.	Fruit immersion in NaHS solution alone or in combination with a NO donor	Accumulation of antifungal enzymes chitinase and beta-glucanase	[120]
Arabidopsis thaliana	*Pseudomonas syringae* pv. *tomato* DC3000	*DES1* and *DCD* overexpression, H_2S donor NaHS*DES1* and *DCD* knock-down, H_2S scavenger hypotaurine	Decreased bacteria count in infected tissuesIncreased bacteria count in infected tissues	[75]
n.a.	*Pseudomonas syringae pv. phaseolicola (Pph) 1302A*	H_2S donors (NaHS, Na_2S, AP39 – mitochondria-targeted H_2S donor)	Inhibition of cell growth, increased virulence	[121]

n.a., not applicable.

result in HS^- protonation and formation of H_2S to re-establish the equilibrium. Some previous studies concluded that amounts of H_2S produced by plants were not sufficient to exert its toxic effect to plant pathogens [25]. H_2S fumigation experiments showed that even relatively high 20 $\mu l \cdot L^{-1}$concentrations of H_2S (i.e. two orders of magnitude higher than levels known to decrease plant growth) reduced the growth of fungal pathogen *Rhizoctonia solani* only by 17%; moreover, prolonged fumigation resulted in increased growth of bacterial colonies [125]. In contrast, other results demonstrated that plant were capable to reduce fungal pathogen growth through localized high H_2S production at the site of infection and on the leaf surface. In *A. thaliana*, H_2S concentrations in leaf mesophyll were reported within the range of 4–10 μM [126]. Still, it has not been decisively demonstrated if pathogen destruction is the primary role of H_2S emission or whether it is just its side effect.

Another unresolved issue concerns the capability of plant-produced H_2S to enter pathogen cells. H_2S can be transported from chloroplasts to the cytosol by directed transport enabled by specific transporter proteins. It is supposed that cytosolic HS^-, representing at pH 7.4 approx. 75% of hydrogen sulfide, can be transported to the apoplastic space, although the HS^- transporters in plant membranes have not been characterized yet [44]. Apoplastic pH is known to increase during plant-pathogen interactions, which can ensure that the equilibrium is shifted toward HS^- anion, thus avoiding diffusion of H_2S back into the plant cells [127]. Nevertheless, the molecular mechanism of how H_2S enters pathogen cells remains unresolved.

Locally increased H_2S concentrations in the site of pathogen attack were suggested to inhibit spore germination or to decrease the growth rate of fungal hyphae. H_2S can be oxidized in presence of electron acceptor or by the catalytic action of superoxide dismutases to elemental sulfur, which is known to be toxic in significantly lower levels compared to H_2S itself [41,88]. Besides its direct toxic effect to plant pathogens, H_2S is involved in the activation of signalling pathways regulating plant responses to pathogen recognition and penetration. Among these mechanisms, protein persulfidation (previously termed also as S-sulfhydration) as a post-translational protein modification can strongly affect protein biological activity [29,39,97]. On reaction with H_2S, persulfidated proteins have cysteine thiol groups modified to –SSH group. In Arabidopsis *des1* mutants, persulfidation levels were changed in important proteins involved in intracellular signalling processes, e.g. ASNF1-related protein kinase 2.2 or the ABA receptor [97].

It can be hypothesized that besides direct toxic effects to microbial cells, molecular mechanisms of H_2S effects in plant-pathogen interaction include numerous chemical reactions with RNS and ROS leading to reactive sulfur species, which mediate protein post-translational modifications like persulfidation (Fig. 2). Persulfidation might be an efficient regulatory mechanism to activate defence responses, including activation of enzymes, ion channels or transcription factors, ultimately leading to activation of defence phytohormone signalling, production of antimicrobial metabolites and establishment of local or systemic resistance.

Conclusions and future perspectives

Precise regulation of L-Cys homeostasis in the cytosol of plant cells by OAS-A1 and DES1 is necessary for plant sulfur metabolism and plant responses to stress conditions. Modulations of enzyme activities of OAS-A1 and DES during plant development and in reaction to environmental conditions regulate the levels of L-cysteine and H_2S [128]. It should be noted that many published reports describing the effects of H_2S in biological systems including plants were obtained using solutions of NaHS or Na_2S as "H_2S donors". In solution, these inorganic sulfides are H_2S equivalents, but their dissolution results in a fast formation of high H_2S levels, unlike in case of synthetic H_2S releasing compounds that can

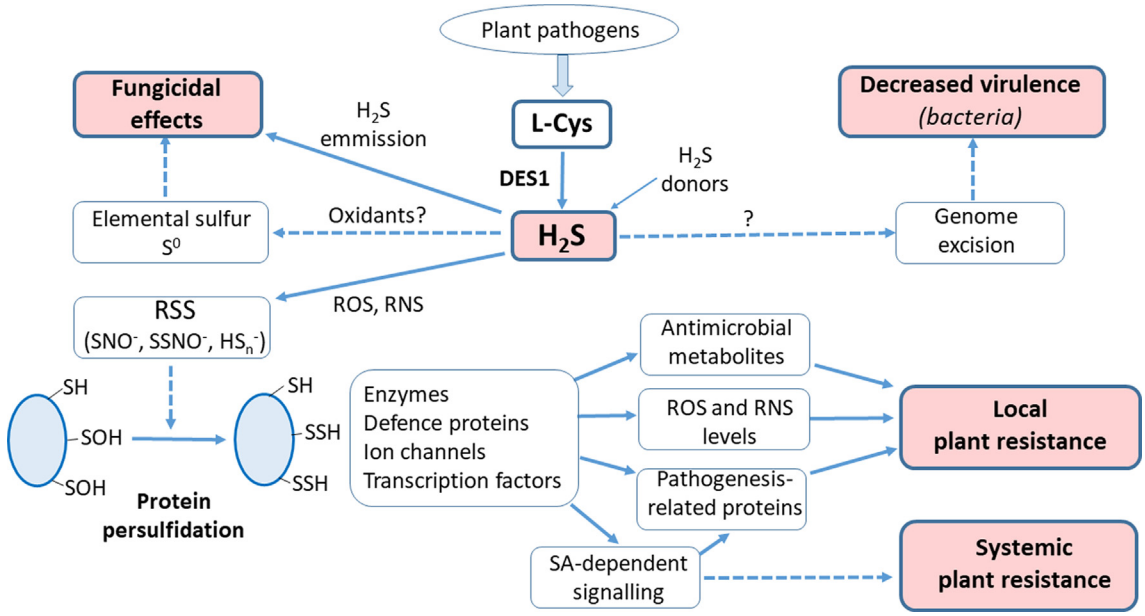

Fig. 2. Schematic overview of known molecular mechanisms of H_2S involvement in increased plant resistance to microbial pathogens. DES1, L-cysteine desulfurase; PR, pathogenesis-related; RNS, reactive nitrogen species; ROS, reactive oxygen species; RSS, reactive sulfur species; SA, salicylic acid; SNO-, thionitrite; $SSNO^-$, perthionitrite; HS_n^-, polysulfides.

mimic low and steady H_2S levels occurring in living tissues. Moreover, in an aerobic environment, NaHS and Na_2S solutions are known to contain numerous sulfur species, including polysulfides, S^0 as well as thiyl radicals as products of sulfide autoxidation [129]. Introduction and validation of reliable methods for the quantitative analysis of H_2S and its metabolites, already widely used within animal H_2S research, is required to solve controversies on biological effects of H_2S in plants under physiological and stress conditions.

In plant systems, rigorous studies focused to the identification of the active agent and analysis of reaction mechanisms have been lacking, including the proposed insertion of sulfur atom(s) into sulfhydryl groups [130,131]. The first report on the Arabidopsis persulfidome established the prominent role of this post-translational modification in plant H_2S signalling [39,97,132]. The recently developed dimedone switch method provided deeper insights into the mechanistic details of protein persulfidation, which occurs on sulfenylated cysteine residues and thus protects proteins from over-oxidation under stress conditions [133]. This mechanism is evolutionary conserved from the bacteria to humans and represent a putative interconnection of signalling pathways of H_2S, ROS and NO through diverse cysteine post-translational modifications; however, if this persulfidation mechanism operates also in plants has not been tested yet. Significant gaps exist in the knowledge of the regulation of endogenous H_2S levels, the sources and their modulation for H_2S signalling as well as the molecular targets of H_2S both in plant and pathogen cells. So to fully understand the regulatory and signalling roles of DES1 and its reaction product H_2S, further systematic studies are required on the sulfur chemistry in plant cell compartments varying in pH values and levels of ROS, thiols and other reaction partners [28,134].

The major part of our actual knowledge on the role of L-Cys and H_2S in plant resistance to phytopathogens has been obtained on model plant species *A. thaliana* using specific mutants with down- or up-regulated enzymes of L-Cys metabolic pathways. Thus further experiments on agriculturally relevant crops, ideally in the field conditions, can contribute to transfer the knowledge on molecular mechanisms of the involvement of sulfur-containing compounds in plant biotic interaction into their practical application towards increased crop resistance. In this regard, new technologies available for direct plant genome editing [135] can be considered a promising tools to further understand plant pathosystems by modulations of genes coding plant proteins and enzymes involved in H_2S metabolism, signalling and defence mechanisms activated upon pathogen challenge.

Compliance with ethics requirements

This article does not contain any studies with human or animal subjects

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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