Isolation and characterization of cysteine-degrading and H_2S-releasing proteins in higher plants

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Summary

The topic of this work is the characterization of cysteine-degrading and H_2S-releasing enzymes in higher plants. The reaction of two candidates, the OAS-TL isoforms and the cysteine desulfhydrases (CDes) have been observed under different conditions. *O*-acetyl-L-serine(thiol)-lyase (OAS-TL) is responsible for the formation of the first stable organic sulfur compound in the cysteine biosynthesis. It catalyses the incorporation of sulfide to the β-position of *O*-acetyl-L-serine (OAS) leading to the formation of cysteine. But OAS-TL is also able to release H_2S in a side reaction. CDes proteins are mainly responsible for the degradation of cysteine to H_2S, pyruvate and ammonium or to alanine and H_2S.

Fumigation experiments with H_2S have shown that increased cysteine and glutathione concentrations by using and/or detoxifing atmospheric H_2S seems to be confirm with enhanced *in vivo* OAS-TL enzyme activity without significantly higher *in vitro* enzyme activity at elevated H_2S levels. Slight decreases in the OAS concentration in the plants support this theory of detoxification because of syntheses or degrading processes.

Experiments with transgenic *OAS-TL* antisense potato plants led to the conclusion that OAS-TL has protected the cysteine homeostasis because OAS-TL was bound in the multi-enzyme complex to inactivate the reverse reaction. Interestingly, in potato plants the H_2S-releasing side seemed to be mainly catalysed OAS-TL enzyme activity.

Experiments done in order to clarify the role of H_2S-releasing enzymes and pathogen attack showed that there seems to be a direct correlation between higher L-cysteine desulfhydrase enzyme activity and pathogen attacks. Independent from the sulfur status of the plants highest *CDes* mRNA accumulation could be observed in stems which would mean that if the precursor for the degradation to H_2S is limited, higher sulfur supply would only enhance the possibility of releasing H_2S. This situation supports the newest discussion where the term sulfur-induced resistance (SIR) should be replaced by sulfur-enhanced defence (SED). Calculated from a fumigation experiment done with *Arabidopsis* plants the H_2S amounts potentially released against pathogens could not be higher than 0,5 µl l^{-1} H_2S. Otherwise the plants suffer oxidative stress. Innovative experiments with a H_2S microsensor were done to detect H_2S concentration in plants *in vivo.*

The identification of D-cysteine desulfhydrases in *Arabidopsis* and the characterization of the enzyme activity of one of them were successfully done. The substrates specify might be an explanation for a purpose separation between D- and L-cysteine degrading CDes.

Keywords: Cysteine desulfhydrases, D- and L-amino acids, microsensor, OAS-TL, SED, SIR

Zusammenfassung

Thema dieser Arbeit ist die Charakterisierung von Cystein-abbauenden und H_2S-freisetzenden Enzymen in Höheren Pflanzen. Dafür sind zwei Enzyme unter unterschiedlichsten Bedingung untersucht worden. *O*-Acetyl-L-Serin(thiol)lyasen katalysieren die Verknüpfung von Sulfid mit *O*-Acetyl-L-Serin (OAS), wobei Cystein entsteht. Allerdings können OAS-TLs auch in einer Seitenreaktion den Abbau von Cystein zu H_2S katalysieren. Für diesen Degradierungsschritt sind hauptsächlich Cystein-Desulfhydrasen (CDes) zuständig, indem sie H_2S, Pyruvat und Ammonium oder Alanin und H_2S freisetzen.

H_2S-Begasungsexperimente mit *Arabidopsis thaliana* haben gezeigt, dass die ansteigende Cystein- und Glutathion-Konzentration anscheinend mit der Nutzung und/oder der Entgiftung des atmosphärischen H_2S durch gesteigerte *in vivo* OAS-TL Enzymaktivität zustande kommt, ohne dass *in vitro* erhöhte OAS-TL Enzymaktivität gemessene werden kann. Die tendenzielle Abnahme von OAS während der Begasung in den Pflanzen unterstützt die Theorie der Entgiftung durch Synthese- oder degradierende Prozesse.

Experimente mit transgenen *OAS-TL* Kartoffel-Pflanzen lassen den Schluss zu, dass OAS-TL für die Erhaltung der Cystein-Homöostase durch die Bindung an den Multi-Enzym-Komplex Cystein-Synthase verantwortlich ist. Gebunden im Komplex ist es für das OAS-TL Enzym nicht möglich, eine Rückreaktion zu H_2S zu katalysieren. Allerdings scheint es so, dass die cytosolische OAS-TL-Isoform maßgeblich an der Freisetzung von H_2S beteiligt ist.

In Experimenten, die unternommen wurden, um die Rolle von H_2S-freisetzenden Enzymen bei Pathogenbefall zu überprüfen, konnte eine direkte Korrelation von erhöhter L-Cystein Desulfhydrase Aktivität mit Pathogenbefall gezeigt werden. Unabhängig vom Schwefel-Status der Pflanzen konnte eine erhöhte Akkumulation von *CDes* mRNA in Stängeln gezeigt werden. Dies würde wiederum bedeuten, dass auch wenn das Substrat für eine H_2S-Freisetzung limitiert ist, Schwefelzugabe nur eine Erhöhung der Möglichkeit zur H_2S-Freisetzung bewirkt. Diese Beobachtung spiegelt die derzeitige Diskussion über eine Begriffsänderung von Schwefel-induzierter Resistenz (sulfur-induced resistence; SIR) zur Schwefel-erhöhten Abwehr (sulfur-enhanced defence; SED) wider. Auf Grund der Ergebnisse in einem H_2S-Begasungs-Versuch, kann spekuliert werden, dass *Arabidopsis*-Pflanzen nur bis zu 0,5 µl l^{-1} H_2S gegen Pathogene einsetzen könnten. Höhere H_2S-Konzentrationen führen zu oxidativem Stress. Die Einbeziehung einer H_2S-Mikroelektrode stellt die innovative Möglichkeit dar, H_2S Konzentrationen *in vivo* zu messen.

Die Identifikation von D-Cystein-Desulfhydrasen in *Arabidopsis* und die Charakterisierung von einem der beiden Enzyme war möglich. Mögliche substratspezifische Aufgabenteilungen

zwischen L-und D-Cystein-degradierenden CDes könnte das Vorhandensein von beiden Enzym-Varianten erklären.

Schlüsselwörter: Desulfhydrasen, D- und L-Aminosäuren, Mikroelekrode, OAS-TL, SED, SIR

Abbreviation

aa	amino acids
ACC	1-aminocyclopropane-1-carboxylic acid
Acc. no.	accession number
AOA	aminooxy acetic acid
At	*Arabidopsis thaliana*
AtStr1	*Arabidopsis thaliana* sulfurtransferase 1
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
Bn	*Brassica napus*
BSA	bovine serum albumine
CAS	*β*-cyano-L-alanine synthase
CDes	cysteine desulfhydrase
CDP	2-chlor-5-(4-methoyxspiro{1,2-dioxetan-3,2'-(5'-chlor)tricycle[3.3.1.13,7]decan}-4-yl-1-phenylphosphate
Cys	cysteine
D-CDes	D-cysteine desulfhydrase
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DTT	dithiothreitol
EC	enzyme classification
EDTA	ethylenediaminetetraacetic acid
EST	expressed sequenced tag
FW	fresh weight
GFP	green fluorescent protein
GSH	glutathione
H_2S	hydrogen sulfide
IPTG	isopropyl thio-β-D-galactoside
KCN	potassium cyanide
LCD	L-cysteine desulfhydrase
L-CDes	L-cysteine desulfhydrase

N	nitrogen
NAS	*N*-acetyl-L-serine
NBT	nitroblue tetrazolium
OAS	*O*-acetyl-L-serine
OASTL	*O*-acetyl-L-serine(thiol)lyase
OAS-TL	*O*-acetyl-L-serine(thiol)lyase
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
PEG	polyethylene glycol
PLP	pyridoxal-5'-phosphate
Rhd	rhodanese
RNA	ribonucleic acid
S	sulfur
SAT	serine acetyltransferase
SED	sulfur-enhanced resistance
SIR	sulfur-induced resistance
St	*Solanum tuberosum*
Wt	wildtype

The following papers cover part of this thesis:

Bloem E, Riemenschneider A, Volker J, Papenbrock J, Schmidt A, Salac I, Haneklaus S, Schnug E. 2004. Sulphur supply and infection with *Pyrenopeziza brassicae* influence L-cysteine desulphydrase activity in *Brassica napus* L. J Exp Bot 55: 2305-2312. Reprinted by permission of Oxford University Press (Chapter 5)

Riemenschneider A, Nikiforova V, De Kok LJ, Papenbrock J. 2005. Impact of elevated H_2S on metabolite levels, activity of enzymes and expression of genes involved in cysteine metabolism. Plant Physiol Biochem 43: 473-483. Reprinted with permission from Elsevier (Chapter 3)

Riemenschneider A, Wegele R, Schmidt A, Papenbrock J. 2005a. Isolation and characterisation of a D-cysteine desulfhydrase protein from *Arabidopsis thaliana*. FEBS J 272, 1291-1304. Reprinted with permission from Blackwell (Chapter 7)

Riemenschneider A, Riedel K, Hoefgen R, Papenbrock J, Hesse H. 2005b. Impact of reduced *O*-acetylserine(thiol)lyase isoform contents on potato (*Solanum tuberosum* L.) plant metabolism. Plant Physiol 137: 892-900. Reprinted with permission from ASPB (Chapter 2)

Riemenschneider A, Bonacina E, Schmidt A, Papenbrock J. 2005. Isolation and characterization of a second D-cysteine desulfhydrase-like protein from *Arabidopsis*. In: Sulfur Transport and Assimilation in Plants in the Post Genomic Era. Saito K, De Kok LJ, Stulen I, Hawkesford MJ, Schnug E, Sirko A, Rennenberg H (Eds.), Backhuys Publishers, Leiden, pp. 103-106. (Chapter 8)

Contents

Chapter 1

General introduction

Short overview of the sulfur metabolism

Sulfur is an essential macroelement for plant growth and has various biological functions. Sulfur is found in the amino acids cysteine and methionine, in the tripeptide glutathione (GSH) and the phytochelatins, in vitamins and cofactors (biotin, thiamine, coenzyme A, and S-adenosyl-methionine). It can also be found in a variety of secondary compounds, i.e. glucosinolates. Sulfur is taken up into roots from the soil as inorganic sulfate. Under normal conditions the rate of uptake and assimilation of sulfur will depend on the requirement for growth, which can be defined as the rate of sulfur uptake and assimilation required per gram plant biomass produced with time (De Kok et al. 2000; 2002b). The sulfur requirement will fluctuate during plant development and may vary between species differing in sulfur need for growth and the potential sink capacity of secondary sulfur compounds. The uptake of sulfate by the roots and its transport to the shoot seem to be major sites of regulation of sulfur assimilation (Hawkesford & Wray 2000; De Kok et al. 2002a). In *Arabidopsis* 12 sulfate transporter genes have been identified (The *Arabidopsis* Genome Initiative 2000), which may be subdivided into four different groups with different affinities to sulfate (reviewed in Yamaguchi & Sano 2001).

After uptake for assimilation, sulfate needs to be activated by ATP sulfurylase to adenosine 5'-phosphosulfate (APS). This reaction is the sole entry step for the metabolism of sulfate. ATP sulfurylase activity is found in chloroplasts and the cytosol (Rotte & Leustek 2000). However, all four ATP sulfurylase genes in *Arabidopsis* are supposed to encode plastidic forms. The cytosolic isoform is presumably encoded by one of the four genes by using a different translational start codon (Hatzfeld et al. 2000). The function of this isoform is still unclear.

The APS reductase catalyses the step to sulfite and the sulfite reductase catalyses the transfer of six electrons from ferredoxin to sulfite to produce sulfide. This enzyme is localized in plastids of both photosynthetic and nonphotosynthetic tissues. In *Arabidopsis* the sulfite reductase is encoded by a single copy (Bork et al. 1998).

The last step of the cysteine biosynthesis is the incorporation of sulfide to the β-position of *O*-acetyl-serine (OAS) leading to the formation of L-cysteine. L-serine acetyltransferase (SAT)

and *O*-acetyl-L-serine(thiol)-lyase (OAS-TL) are responsible for the formation of the first stable organic sulfur compound. In *Arabidopsis*, OAS-TL proteins are encoded by a small gene family of 4 genes (Jost et al. 2000). The nuclear-encoded OAS-TL isoforms remain either in the cytoplasm (OAS-TL A) (Hell et al. 1994) or are imported into plastids (OAS-TL B) and mitochondria (OAS-TL C), respectively (Hesse et al. 1999). Also SAT isoforms have been found in these three compartments from various plants (cited in Hell 1997).

SAT and OAS-TL are associated in a multienzyme complex called cysteine synthase, first described in *Salmonella typhimurium* and *Escherichia coli* (Kredrich 1996) and later in *Arabidopsis* (Bogdanova et al. 1997; Hell et al. 1994). SAT is only active when bound in the complex. It catalyses the formation of OAS from serine and acetyl-CoA. Since this reaction connects nitrogen and sulfur metabolism, OAS plays a regulatory function in sulfur assimilation (Giovanelli 1990; Leustek & Saito 1999). OAS-TL is virtually inactive in the complex and causes the stabilization of SAT. With increasing OAS amounts the binding affinity of both enzymes decreases and OAS-TL is released to catalyse the incorporation of sulfide to the β-position of OAS leading to the formation of cysteine (Hell et al. 2002).

The role of *O*-acetyl-serine(thiol)lyases and cysteine desulfhydrases in H_2S release

Since the Clean Air Acts have led to a drastic decrease of SO_2 emissions in Western Europe (Dämmgen et al. 1998), macroscopic sulfur deficiency has become the most widespread nutrient disorder since then (Schnug & Haneklaus 1998). Negative impacts on crop quality and yield in general and higher susceptibility of *Brassica napus* crops against certain diseases have been observed (Schnug et al. 1995a). Different field surveys have shown that sulfur fertilization can increase the resistance of agricultural crops against fungal pathogens. The mechanisms of a sulfur-induced resistance (SIR) are, however, not yet known. Volatile sulfur compounds are thought to play an important role because H_2S may be toxic to fungi. At the moment it is not known whether the H_2S emission takes place before or after the cysteine synthesis or whether the H_2S emission is genetically controlled or directly correlated with the sulfur supply. In addition to volatile sulfur compounds the accumulation of elemental sulfur in the veines and vascular tissues might also be involved in resistance against pathogens (Cooper et al. 1996; Williams et al. 2002). Glucosinolates, best-known example of a performed defence compound, and sulfur-containing phytoalexins are also candidates which might react against several pathogens (Smith & Kirkegaard 2002; van Wees et al. 2003).

It is known that in higher concentration cysteine is cytotoxic. Therefore a close regulation is necessary. The cysteine pool size might be controlled by the cysteine biosynthesis or the

control also can be achieved by cysteine-degrading proteins. Is H_2S release only a side product or a waste product because of the cysteine pool regulation? Or does a plant release H_2S actively e.g. as a defence compound?

Because of the chemistry of the cofactor pyridoxal-5'-phosphate (PLP) these enzymes are investigated. In all reactions, the coenzyme PLP acts as an electron sink, storing electrons from cleaved substrate bonds and dispensing them for the formation of new linkages with incoming protons or second substrates. On the basis of available structural information, the B_6 enzymes can be subdivided into four independent families of paralogous groups: the α-family with aspartate aminotransferase as the prototype enzyme, the β-family with tryptophan synthase β as the prototype enzyme, the D-alanine aminotransferase family, and the alanine racemase family. The α-family is the largest one and includes gene products that catalyse reactions in the biosynthesis of deoxyamino and dideoxy sugars with either PLP or pyrodoxamine-5'-phosphate as a prosthetic group. Cysteine desulfhydrases (CDes) and 1-aminocyclopropane-1-carboxylate (ACC) synthase, for example, belong in this family. The β-family consists of lyases, catalysing reactions in which not only C_α but also C_β participate in the covalency changes. OAS-TL proteins catalyse the described α, β replacement reactions (Christen & Mehta 2001).

In *Arabidopsis* seven different genes of the β-substituted alanine-synthase family (*Bsas*) coding for proteins with OAS-TL activity are known (Hatzfeld et al. 2000). The PLP-dependent OAS-TL proteins catalyse the insertion of H_2S into OAS in an α, β-replacement reaction to yield the amino acid L-cysteine (Fig. 1). But OAS-TLs are also involved in the release of H_2S in a side reaction (Burandt et al. 2002; Schmidt 1977a, b).

In *Arabidopsis* mitochondria there exists a true OAS-TL and a β-cyano-L-alanine-synthase (CAS). In higher plants CAS plays a role in cyanide detoxification (Yamaguchi et al. 2000). It catalyses the formation of sulfide and β-cyano-L-alanine from cysteine and cyanide (Blumenthal et al. 1968). In a side reaction CAS is able to catalyse the incorporation of sulfide into cysteine (Hatzfeld et al. 2000; Jost et al. 2000). Interestingly, in potato (*Solanum tuberosum*) and in spinach (*Spinacia oleracea*) mitochondria only CAS but no OAS-TL can be found. According to the favourable conditions of the non-existing third OAS-TL isoform in mitochondria, potatoes are good candidates to promote the understanding of the role of the different OAS-TL isoforms and their role in H_2S-release. For this purpose transgenic potato plants via antisense mediated inhibition have been produced (Hesse & Höfgen 1998).

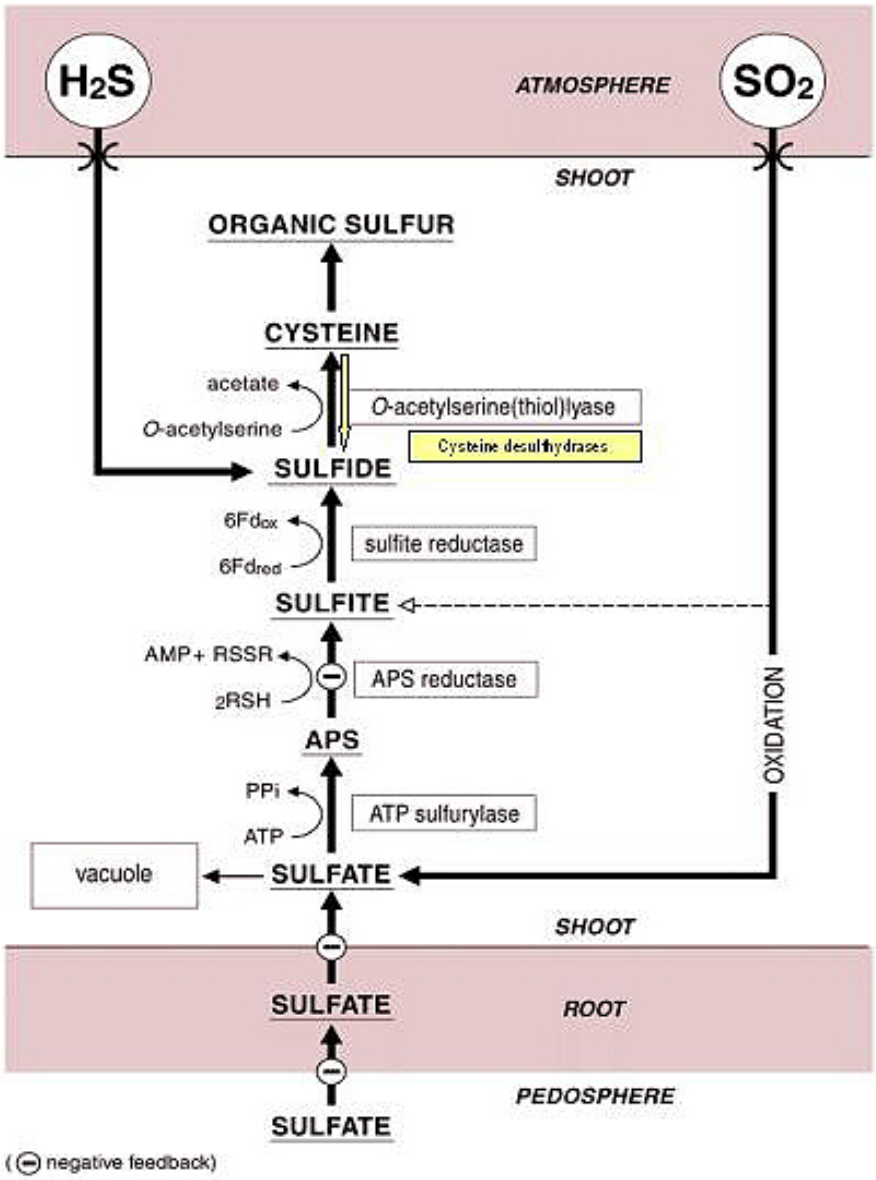


Fig 1: Sulfate assimilation pathway in higher plants. The pathway shows the uptake of sulfate and its reduction. The sulfite reductase is only localised in plastids. H_2S could be produced via reduction or is taken up from the atmosphere. *O*-acetyl-serine(thiol)lyases catalyse the incorporation of sulfide to cysteine. APS, adenosine 5'-phosphosulfate; RSH, RSSG, thiols; fd, ferredoxin. ɵ possible sites of feedback inhibition of sulfate uptake. Degradation of cysteine might be catalysed by cysteine desulfhydrases (adapted from De Kok et al. 2002b).

Several PLP-dependent L-cysteine desulfhydrases (L-CDes) are known as essential enzymes required in all living organisms for the degradation of cysteine to yield L-alanine and reduced sulfur for iron-sulfur (Fe-S) cluster biosynthesis. Most research on Fe-S cluster assembly has come from studies on bacteria; three bacterial systems exist termed NIF (nitrogen fixation), ISC (iron-sulfur cluster), and SUF (mobilization of sulfur) (Zheng et al. 1998; Takahashi & Tokumoto 2002; Loiseau et al. 2003). The NIF system is most specific, involved in the

assembly and maturation of Fe-S clusters in nitrogenase proteins in nitrogen-fixing bacteria and ε-proteobacteria (Jacobson et al. 1989). The cysteine desulfhydrase activity was first described for a NifS protein from *Azotobacter vinelandii* (Zheng et al. 1993). The ISC system is more general and is found in both bacteria and higher eukaryotes (Zheng et al. 1998; Muhlenhoff & Lill 2000; Gerber & Lill 2002). The *suf* operon (*sufABCDSE*) represents the third system involved in Fe-S cluster biogenesis (Takahashi & Tokumoto 2002; Loiseau et al. 2003; Ollagnier-de-Choudens et al. 2003).

L-cysteine desulfhydrases from plants catalyse the degradation of cysteine to pyruvate, ammonium and H_2S or to alanine and H_2S (Rennenberg et al. 1987). Recently, in plants a NifS-like protein belonging to the NIF/ISC Fe-S cluster biogenesis system was identified in *Arabidopsis* localized in the mitochondrion (AtNFS1). A second protein has been found localised in chloroplasts with high homology to SufS, part of the SUF system. Because of independent researches it is named AtNFS2/AtCpNifS (Kushnir et al. 2001; Leon et al. 2002; Pilon-Smits et al. 2002). By the degradation of L-cysteine alanine was formed (Pilon-Smits et al. 2002).Because of using L-selenocysteine as substrate the authors speculate that AtNFS2/AtCpNifS (SufS) could also play a role in seleno-protein synthesis.

Burandt et al. (2001c) observed that the specific L-cysteine desulfhydrase activity increased with increasing plant age. It is also possible to conclude for *Brassica napus* that the higher the sulfur content in the plants was, the lower the activity of the H_2S-releasing enzyme L-CDes. A genetic influence on the activity of the investigated total L-CDes enzyme extract might be probable (Burandt et al. 2001a).

The aim of this work is to discover which role the cysteine desulfhydrases play in the release of H_2S acting against pathogens. One problem of estimating the results is that plants are also able to use H_2S as sulfur supply from the atmosphere. H_2S is taken up via stomata and is metabolised with high affinity into cysteine. After a short period of exposure to H_2S sulfate uptake is reduced in the roots (Westerman et al. 2000).

Because of rather limited studies on the model plant *Arabidopsis* effects of atmospheric H_2S will be investigated. This experiment might help to understand the regulatory mechanisms of the maintenance of the cysteine pool size and metabolism influenced by increased levels of atmospheric H_2S. The expression analysis of the OAS-TL isozymes during H_2S fumigation might clarify the role of different cellular compartments, the cytoplasm, mitochondria, and plastids, in assimilatory and dissimilatory sulfur metabolism.

To investigate and assess the involvement of H_2S in SIR it would be very important to determine the internal H_2S concentration. For such measurements it is planned to use a sulfide

microsensor to measure H_2S release from leaves and the H_2S concentration in different compartments of the leaf.

The integration of field experiments will help to evaluate the relevance of sulfur nutrition on SIR under field conditions. In this field experiment two different varieties of oilseed rape (*B. napus*) will be infected with *Pyrenopeziza brassicae*. Different levels of nitrogen and sulfur supply will be used. The relationship between the nutritional status of oilseed rape compared with the fungal infection and the H_2S releasing enzyme activity are of main interest.

Detailed studies with *B. napus* will be done to investigate the influence of desulfhydrases in the release of H_2S acting against pathogens.

The role of D-cysteine desulfhydrases in H_2S release

Generally, amino acids (aa) are used in the L-form, and enzymes involved in their metabolism are stereospecific for the L-form. With some exceptions, each amino acid of importance has one asymmetric C atom that can exist in two mirror images (L- or D-conformation), called enantiomers. Interestingly, D-amino acids are widely distributed in living organisms (Friedman 1999). In plants D-amino acids were detected in gymnosperms, in mono- and dicotyledonous angiosperms via HPLC or GC-MS. Free D-amino acids in the low percentage range of 0,5 to 3% relative to their L-enantiomers are principle constituents of plants (Brückner & Westerhauser 2003). However, the functions of D-amino acids and their metabolism are largely unknown.

Focusing on our interests in PLP-dependent enzymes only a few PLP enzymes that act on D-amino acids have been found such as D-serine dehydratase (Dowhan & Snell 1970), 3-chloro D-alanine chloride-lyase (Nagasawa et al. 1982), and D-cysteine desulfhydrase (D-CDes) (Nagasawa et al. 1985; 1988; Schmidt 1982).

The *Escherichia coli* (D-CDes) (EC 4.1.99.4) a PLP-containing enzyme was detected which is capable of catalysing the α,β-elimination reaction of D-cysteine into pyruvate, H_2S, and ammonium. It also catalyses the transformation of several D-cysteine derivatives and the formation of D-cysteine or D-cysteine-related aa from β-chloro-D-alanine in the presence of various thiols or from *O*-acetyl-D-serine and H_2S (Nagasawa et al. 1985; Nagasawa et al. 1988). The physiological role of bacterial D-CDes is unknown. To learn more about the role of D-CDes in adaptation to D-cysteine, the gene was cloned from *E. coli* corresponding to the ORF *yedO* at 43.03 min on the genetic map of *E. coli* (Soutourina et al. 2001). In the presence of the *yedO* gene D-cysteine as sole sulfur source stimulates cell growth because the

bacterium is able to utilise H_2S released from D-cysteine as sulfur source; the protein exhibited D-cysteine desulfhydrase activity.

In several organisms a D-cysteine desulfhydrase activity could be observed. *Spinacia oleracea*, *Chlorella fusca*, *Cucurbita pepo*, *Cucumis sativus* and suspension cultures of *Nicotiana tabacum* (Schmidt 1982; Schmidt & Erdle 1983; Rennenberg 1983a; Rennenberg et al. 1987) are examples where it was possible to clearly separate the D-cysteine desulfhydrase activity from L-cysteine desulfhydrase activity by demonstrating different pH optima for the enzyme activity (Schmidt 1982), different sensitivity to inhibitors (Rennenberg et al. 1987), and different localization in the cell (Rennenberg et al. 1987). Both CDes protein fractions were separated by conventional column chromatography, however, because of low protein stability and low yields neither of the proteins could be purified to homogeneity from plant material (Schmidt 1982; Schmidt & Erdle 1983). After the results of Soutourina et al. (2001) who could show D-cysteine desulfhydrase activity of the YedO protein an identification of an *Arabidopsis* homologue (At1g48420) to *yedO* was possible. The deduced protein might be a good candidate for the first D-CDes enzyme in higher plants of which the sequence is known.

The aim of the thesis

- Analyses of *OAS-TL* antisense potato plants to asses the function of the OAS-TL isoforms and their influence on H_2S-releasing capacity.

- Fumigation of *Arabidopsis* plants with different concentrations of H_2S to investigate its influence on the expression of OAS-TLs and desulfhydrases and their enzyme activity.

- Measurements with a sulfide microsensor at infected and noninfected plant material to find out the H_2S concentration in plants and the physiological H_2S emission of plants.

- Field experiment with *Brassica napus* infected with *Pyrenopeziza brassicae* to learn more about nutrient-mediated plant health and its influence on SIR.

- Additional experiments with *Brassica napus* revealing the desulfhydrases' expression pattern under sulfur starvation and pathogen attack.

- Characterization of putative D-cysteine desulfhydrases in *Arabidopsis*.

Chapter 2

Impact of reduced *O*-acetyl-serine(thiol)lyase isoform contents on potato plant metabolism

Plant cysteine (Cys) synthesis can occur in three cellular compartments: the chloroplast, cytoplasm, and mitochondrion. Cys formation is catalysed by the enzyme *O*-acetylserine(thiol)lyase (OASTL) using *O*-acetylserine (OAS) and sulfide as substrates. To unravel the function of different isoforms of OASTL in cellular metabolism, a transgenic approach was used to downregulate specifically the plastidial and cytosolic isoforms in potato (*Solanum tuberosum*). This approach resulted in decreased RNA, protein, and enzymatic activity levels. Intriguingly, H_2S-releasing capacity was also reduced in these lines. Unexpectedly, the thiol levels in the transgenic lines were, regardless of the selected OASTL isoform, significantly elevated. Furthermore, levels of metabolites such as serine, OAS, methionine, threonine, isoleucine, and lysine also increased in the investigated transgenic lines. This indicates that higher Cys levels might influence methionine synthesis and subsequently pathway-related amino acids. The increase of serine and OAS points to suboptimal Cys synthesis in transgenic plants. Taking these findings together, it can be assumed that excess OASTL activity regulates not only Cys de novo synthesis but also its homeostasis. A model for the regulation of Cys levels in plants is proposed.

Introduction

Cys is the first committed molecule in plant metabolism that contains both sulfur and nitrogen, and, thus, the regulation of its biosynthesis is of utmost importance for the synthesis of a number of essential metabolites in plant pathways (Hesse et al. 2004). Cys is incorporated into proteins and glutathione (GSH) directly or serves as a sulfur donor for the synthesis of S-containing compounds such as Met and its derivatives S-adenosylmethionine and S-methylmethionine, and many secondary compounds such as S-methylcysteine, S-alkylcysteine, glucosinolates, and phytoalexins (Schmidt & Jäger 1992; Ravanel et al. 1998; Matthews 1999; Hesse & Höfgen 2003). Furthermore, Cys acts as a general catalyst in redox reactions through the nucleophilic properties of its sulfur atom, utilizing dithiol-disulfide interchange, as displayed in the thioredoxin and the glutaredoxin systems (Schürmann & Jacquot, 2000; Jacquot et al. 2002). Cys is formed from two substrates, sulfide and activated

Ser, as a carbon backbone and is catalysed by the enzyme *O*-acetylserine(thiol)lyase (OASTL), which transfers sulfide to *O*-acetylserine (OAS) to form Cys. The activated Ser, OAS, is synthesized by Ser acetyltransferase (SAT). In plants, OASTL has been shown to be present in 100- to 400-fold excess over SAT (Schmidt & Jäger 1992; Höfgen et al. 2001). There is biochemical and molecular evidence that in plants, SAT and OASTL are associated in a multienzyme complex called Cys synthase, first described in *Salmonella typhimurium* and *Escherichia coli* and later in *Arabidopsis* (*Arabidopsis thaliana*; Kredich 1996; Bogdanova et al. 1997; Wirtz et al. 2001; Berkowitz et al. 2002). The current model of Cys formation proposes that in the formed complex of OASTL and SAT, OASTL is virtually inactive but causes the stabilization of SAT, while SAT is only active when bound in the complex. OAS formed in the complex now decreases the binding affinity of both enzymes, and OASTL is released to convert OAS to Cys; thus, the dissociation serves to control OAS synthesis. However, the concentration of OASTL is far in excess of SAT, and the free OASTL is responsible for the production of Cys (Hawkesford 2000; Saito 2000; Berkowitz et al. 2002; Hell et al. 2002). Cys synthesis occurs at several subcellular locations, each of which has its own enzyme isoforms, at least in *Arabidopsis* with its gene family consisting of seven isoforms (Jost et al. 2000; Höfgen et al. 2001). There, the presence of isoforms in the cytosol, the plastids, and mitochondria suggests that the ability to form Cys is essential for all compartments active in protein biosynthesis. However, their respective contributions to the net Cys synthesis and any functional interactions that may occur between these subcellular locations are unknown. Interestingly, only *Arabidopsis* seems to possess a mitochondrial localized OASTL (Hesse et al. 1999), while in other plants such as spinach (*Spinacia oleracea*) and potato (*Solanum tuberosum*), β-cyanoalanine synthase (CAS) substitutes for this function (Fig. 1; Saito et al. 1994; Warrilow & Hawkesford 1998, 2000; Hatzfeld et al. 2000; Maruyama et al. 2001). In this context, it is important to note that in *Arabidopsis* mitochondria, a CAS exists additionally to OASTL (Hatzfeld et al. 2000). To promote our understanding of the role of the different OASTL isoforms, we initiated a transgenic approach of producing potato plants with reduced enzymatic activities for OASTL via antisense-mediated inhibition of previously cloned endogenous potato OASTL isoforms (Hesse & Höfgen 1998). Several plants with respectively decreased activity could be identified. Interestingly, these plants showed no gross alterations of growth or yield under normal growth conditions. It could be demonstrated that the reduction of OASTL enzyme activity in transgenic potato plants resulted in increased thiol levels. These data provide evidence that both isoforms are able to substitute for each other in function, probably due to the transfer of

S intermediates between cellular compartments, and indicate that excess OASTL is responsible for the regulation of Cys homeostasis.

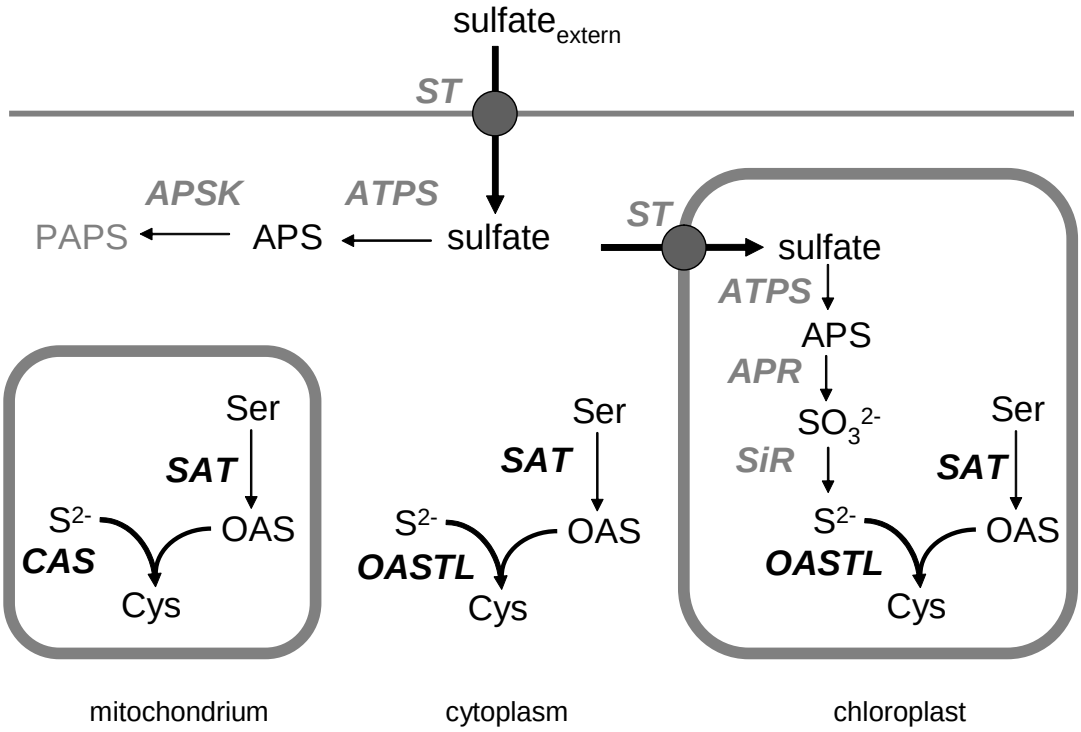


Fig. 1: Sulfur metabolism compartmentation of Cys biosynthesis. External sulfate is taken up through members of a multigene family of sulfate transporters. The inert sulfate is activated by covalent binding to ATP to form adenosine-5'-phosphosulfate (APS) either in the cytosol or plastid. In the cytosol, APS can be phosphorylated to phosphoadenosine-5'-phosphosulfate (PAPS). In chloroplasts, sulfate bound in APS is reduced to sulfide via sulfite and subsequently transferred to activated Ser (OAS) to form Cys. Cys formation takes place in three cellular compartments: chloroplasts, but also cytosol and mitochondria. In these compartments, both SAT and OASTL isoforms are present but the reductive component of the pathway is missing. In potato, the function of OASTL in mitochondria is probably substituted by CAS. ATPS, ATP sulfurylase; APSK, APS kinase; APR, APS reductase; ST, sulfate transporter; SiR, sulfite reductase; ser, serine; S^{2-}, sulfide; SO_3^{2-}, sulfite.

Materials and methods

Generation of transgenic potato lines

Potato (*Solanum tuberosum*; Saatzucht Lange AG, Bad Schwartau, Germany) OASTL isoforms (Hesse & Höfgen 1998) were cut from the pBluescript SK2 as *Asp*718/*Bam*HI fragment for the cytosolic OASTL isoform (1.308 bp; as*OAS-TL A*) and the *Xho*I/*Bam*HI fragment for the plastidial OASTL isoform (1.404 bp; as*OAS-TL B*) and cloned in their reverse orientations to the cauliflower mosaic virus 35S promoter into the vector pBinAR-Kan (Höfgen & Willmitzer 1990) previously cut with *Asp*718/*Bam*HI and *Bam*HI/*Sal*I, respectively, to generate antisense constructs for plant transformation. The transformation of potato by *Agrobacterium tumefaciens* (Rocha-Sosa et al. 1989) using the strain C58C1/pGV2260 (Deblaere et al. 1985) was carried out as described by Dietze et al. (1995). Transgenic plants were selected on medium containing kanamycin (10 mg l^{-1}). The resulting transgenic plants were planted in soil and grown in the greenhouse under a 16-h-light, 8-h-dark regime at 20°C. Leaf material was screened for OASTL activity according to Gaitonde (1967).

Plant cultivation

Transgenic OASTL antisense plants were propagated in tissue culture along with potato wild-type plants and transferred into soil after 2 weeks of cultivation. The rooted shoots were planted in small pots and grown in the phytotron with a light regime of 200 to 250 µmol*s^{-1}*m^{-1} (16 h/8 h) under a hood to retain high air humidity. After 2 weeks, plants were transferred into pots with a diameter of 20 cm and cultivated in a greenhouse providing nearly natural light conditions with an approximately 16-h-light/8-h-dark period plus natural sunlight. Light intensity and temperature were dependent on environmental conditions, but light did not fall below 250 to 300 µmol photons*m^{-1}*s^{-1}, and temperature did not sink below 18°C. Leaf material was harvested from greenhouse-grown plants after approximately 8 weeks of cultivation, before the onset of flowering. Leaf discs were excised from tissues of similar developmental stage. Transition to the reproductive stage could usually be observed only in plants older than 10 weeks. All plant material was sampled in the morning and immediately frozen in liquid nitrogen before storage at -80°C.

RNA extraction and Northern blot analysis

Total RNA was extracted essentially as described by Chomczynski & Sacchi (1987). RNA samples (15 µg) were separated on 1% denaturing agarose-formaldehyde gels. Equal loading

was controlled by staining the gels with ethidium bromide. After RNA transfer onto nylon membranes, filters were probed with digoxigenin-labeled cDNA fragments obtained by PCR encoding the respective mature OASTL proteins. For the PCR-labeling reaction of OASTL A, primer P95 (5'-GGATCCGCGGGGGAAAAGAATGGAA-3') and P96 (5'-GACGTCTCAAGGCTCCACAGTCAT-3') were used. For OASTL B, primer P97 (5'-GGATCCGCAGTGTCTGTACCAACGAAA-3') and P98 (5'-GACGTCTCACAATTCTGGCTTCAT-3') with the respective cDNA clone as template were used (Hesse & Höfgen 1998). A colorimetric detection method with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate as substrates for alkaline phosphatase was applied.

SDS-PAGE and Western blotting

For the determination of protein steady-state levels, 100 mg plant material was mortared to a fine powder in liquid nitrogen. Protein estimation was done according to Bradford (1976) using bovine serum albumin as a standard. A total of 500 µl of sample buffer (56mM Na_2CO_3, 56mM dithiothreitol, 2% SDS, 12% Suc, and 2 mM EDTA) was added, samples were incubated for 15 min at 95°C, and cell debris was removed by centrifugation. About 10 µg of the total protein supernatant was subjected to SDS-PAGE (Laemmli 1970) and blotted (Sambrook et al. 1989). Antibodies directed against purified spinach (*Spinacia oleracea*) OASTL, purified spinach CAS (Hatzfeld et al. 2000), and SAT (Saito et al. 1995) were used for the immunodetection. It is not known which OASTL/CAS isoforms were used for raising the antibodies because the N-terminal amino acid sequences of the purified proteins used for immunization have not been determined. A colorimetric detection method with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate as substrates for alkaline phosphatase was applied.

Activity staining

Extracts of soluble proteins were prepared using 100 mg frozen plant material and 250 µl 20-mM Tris-HCl, pH 8.0. Of each sample, 150 µg protein was separated by native PAGE (8%) at a constant current of 25 mA at 4°C. After electrophoresis, the gel was immersed for 3 min at 30°C in the reaction mix (2.5 mM KCN, 2.5 mM L-Cys, and 25 mM CAPS buffer, pH 10.0), which had been prewarmed at 30°C. The incubation was stopped by adding 0.2 mM lead acetate, pH 4.0. The appearance of brown bands revealed the position of the enzymes in the gel (Akopyan et al. 1975).

Determination of enzyme activity

Extracts of soluble proteins were prepared using 100 mg frozen plant material and 1 ml 20-mM Tris/HCl, pH 8.0. The mixture was further homogenized and centrifuged for 10 min at 13.000g. The assay for OASTL activity contained in a total volume of 1 ml: 5 mM OAS, 5 mM Na_2S, 33.4 mM dithiotreitol, 100 mM Tris/HCl, pH 7.5, and 50 µl enzyme extract (Schmidt 1990). The reaction was initiated by the addition of Na_2S and incubated for 30 min at 37°C after which the reaction was terminated by adding 1 ml acidic ninhydrin reagent (0.8% ninhydrin [w/v] in 1:4 concentrated HCl:HOAc) to determine the Cys concentration (Gaitonde 1967). The samples were heated at 100°C for 10 min to allow colour development and cooled on ice. The colour complex was stabilized by adding 900 µl 70% ethanol to 100-ml samples, then the A_{560}. Solutions with different concentrations of Cys were prepared, treated in the same way as the assay samples, and used for the quantification of the enzymatically formed Cys. L-cysteine desulfhydrase activity was measured by the release of sulfide from Cys. The assay for measuring L-Cys desulfhydrase activity contained in a total volume of 1 ml: 100 mM Tris-HCl, pH 8.0, 2.5 mM dithiothreitol, 0.8 mM L-Cys, and 100 µl enzyme extract. After 15 min at 37°C, the reaction was terminated by adding 100 µl of 30 mM $FeCl_3$ dissolved in 1.2 N HCl and 100 µl 20 mM *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride dissolved in 7.2 N HCl (Siegel 1965). The formation of methylene blue was determined at 670 nm in a spectrophotometer. Solutions with different concentrations of Na_2S were prepared, treated in the same way as the assay samples, and used for the quantification of the enzymatically formed H_2S.

Extraction and analysis of soluble thiol compounds

Individual soluble thiols were determined as the sum of their reduced and oxidized forms. One hundred milligrams of fresh ground leaf material was added to 100 mg of polyvinylpolypyrrolidone (previously washed with 0.1 M HCl) and 1 ml of 0.1 M HCl. The samples were shaken for 60 min at room temperature. After centrifugation (15 min at 13.000g; 4°C), the supernatants were frozen at -20°C until reduction/derivatization. Thiols were reduced by incubation with 10 mM dithiothreitol for 40 min at room temperature and derivatized for 15 min in the dark according to Hell & Bergmann (1990) or Kreft et al. (2003). Column eluent was monitored by fluorescence detection (λ_{ex} 380/λ_{em} 480).Mixed standards treated exactly as the sample supernatants were used as a reference for the quantification of Cys and GSH content.

OAS measurement by GC-MS

For GC-MS analysis, polar metabolite fractions were extracted from 60 mg frozen plant material and ground to a fine powder with MeOH/$CHCl_3$. The fraction of polarmetabolites was prepared by liquid partitioning into water as described earlier (Roessner et al. 2000; Wagner et al. 2003). Metabolite samples were derivatized by methoxyamination, using a 20-mg ml^{-1} solution of methoxyamine hydrochloride in pyridine, and subsequent trimethylsilylation, with *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide, (Roessner et al. 2000). A C_{12}, C_{15}, C_{19}, C_{22}, C_{28}, C_{32}, C_{36} *n*-alkane mixture was used for the determination of retention time indices (Wagner et al. 2003). Ribitol was added for internal standardization. Samples were injected in splitless mode (1 µl/sample) and analysed using a quadrupole-type GC-MS system (MD800, ThermoQuest, Manchester, UK). The level of OAS was determined as relative-response ratios of peak areas of this compound to peak area of internal standard (ribitol), normalized with respect to the fresh weight of the sample. The chromatograms and mass spectra were evaluated using MASSLAB software (ThermoQuest).

Extraction and analysis of soluble amino acids

Soluble amino acids were determined following a modified protocol from Scheible et al. (1997). Leaf tissues (about 100 mg/plant) were ground to a fine powder in liquid nitrogen in a bead mill and extracted 3 times for 20 min at 80°C: once with 400 ml of 80% (v/v) aqueous ethanol (buffered with 2.5 mM HEPES/KOH, pH 7.5) and 10 µl of 20 µmol l-nor-Val (as an internal standard), once with 400 ml of 50% (v/v) aqueous ethanol (buffered as before), and once with 200 µl of 80% (v/v) aqueous ethanol. Between the extraction steps, the samples were centrifuged for 10 min at 13.000g, and the supernatants were collected. The combined ethanol/water extracts were stored at -20°C or directly subjected to reversed phase-HPLC using an ODS column (Hypersil C18; 150- x 4.6-mm i.d.; 3 µm; Knauer, Berlin) connected to an HPLC system (Dionex, Idstein, Germany). Amino acids were measured by precolumn derivatization with orthophthaldehyde in combination with fluorescence detection (Lindroth & Mopper 1979) as described by Kreft et al. (2003). Peak areas were integrated using Chromeleon 6.30 software (Dionex) and subjected to quantification by means of calibration curves made from standard mixtures.

Results

Engineering and screening of plants with reduced OASTL enzyme activity

To decrease the activity of OASTL, potato plants were transformed with the vector pBinAR harbouring a cDNA encoding a sequence from either the *StOASTL A* or the *StOASTL B* gene (Hesse & Höfgen 1998) in reverse orientation with respect to the cauliflower mosaic virus 35S promoter. After regenerating 80 independent transgenic plants/construct, lines were selected with respect to reduced OASTL activity and H_2S-releasing activity levels. Eight lines were chosen for detailed analysis based on their reduced activity levels (Fig. 2A). Greenhouse-grown plant material was evaluated and scored based on macroscopic phenotypic alterations. Transgenic plants were phenotypically indistinguishable from wild-type plants (data not shown). OASTL activity was determined in crude protein extracts of the transgenic lines 1, 9, 24, 34, and 38 expressing the plastidial antisense construct and in transgenic lines 2, 3, and 150 expressing the cytosolic antisense construct and compared with control. Wild-type extracts revealed an OASTL activity in crude extracts of about 220 nmol (milligrams protein x minute)$^{-1}$. For the plastidial antisense lines, the activities were down to 150 nmol (milligrams protein x minute)$^{-1}$ (line 38), 160 nmol (milligrams protein x minute)$^{-1}$ (line 34), 170 nmol (milligrams protein x minute)$^{-1}$ (lines 1 and 9), and 210 nmol (milligrams protein x minute)$^{-1}$ (line 24; Fig. 2A). A higher reduction was observed for cytosolic antisense lines, down to 10 nmol (milligrams protein x minute)$^{-1}$ (line 3), 15 nmol (milligrams protein x minute)$^{-1}$ (line 2), and 20 nmol (milligrams protein x minute)$^{-1}$ (line 150). The relative decrease of OASTL in plastidial antisense lines as compared with wild type is clearly smaller than in transgenic plants expressing the cytosolic antisense construct. This observation might indicate the different amounts of enzyme in the different cellular compartments or that the reduced plastidial activity is compensated in part by other OASTL isoforms.

L-Cys desulfhydrase activity was determined in the same extracts of soluble proteins used for the measurement of OASTL activity as described in Figure 2A. The L-Cys desulfhydrase activity was determined by measuring the formation of H_2S from Cys (Fig. 2B). In parallel to the reduction of OASTL activity in the *OASTL* antisense plants, L-Cys desulfhydrase activity was reduced. In the transgenic plants carrying the *OASTL* antisense construct against the cytosolic OASTL, the effects were more pronounced than in the plastidial antisense plants. It was shown previously in *in vitro* experiments that the recombinant purified OASTL B-protein catalysed the formation of Cys from OAS and H_2S, but also the formation of H_2S from Cys. In a molar ratio, the enzyme formed about 25 times more Cys than H_2S per milligram protein during the same incubation time, suggesting H_2S release as a side reaction of the Cys synthase

reaction (Burandt et al. 2001c). Thus, *in vitro*, the reaction of OASTL is a net H_2S-consuming reaction. The results shown in Figure 2B indicate that, also *in vitro*, the OASTL proteins catalyse the release of H_2S from Cys. However, in comparison to the reduction in OASTL activity (Fig. 2A), the level of reduction in H_2Sreleasing activity is not as strong. Therefore, one can assume that H_2S-releasing activity resulted not exclusively from the side activity of OASTL proteins but additionally from other enzymes such as true L-Cys desulfhydrase proteins. The results indicate that a L-Cys desulfhydrase protein is also localized in the cytosol. To further test whether the reduced OASTL activity resulted from a decreased endogenous transcript amount, total RNA from the selected plants was isolated and screened for OASTL expression. For all investigated transgenic lines, a substantial reduction in their transcript levels in comparison to wild-type plants was observed, while RNA levels of the second, not antisensed, isoform were not affected by the manipulation (Fig. 3).

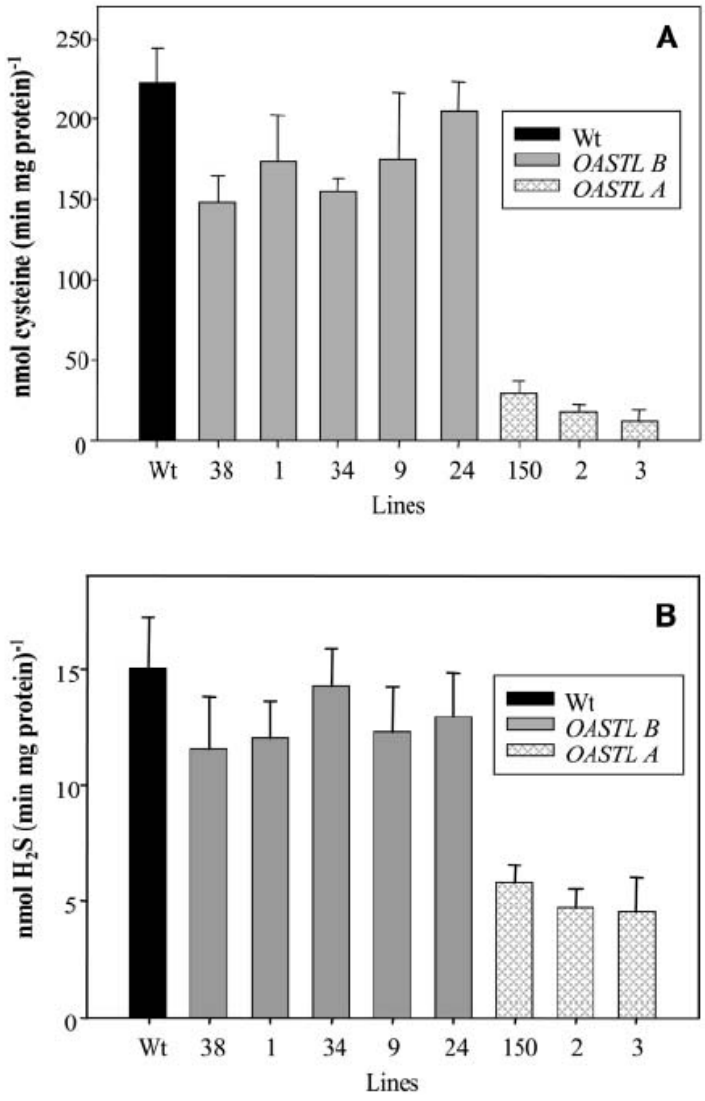


Fig. 2: OASTL and L-Cys desulfhydrase activity in transgenic potato plants. Total extracts of soluble proteins were prepared and the OASTL (A) and L-Cys desulfhydrase (B) activities were determined in spectrophotometric assays by measuring either the formation of Cys (A) or by measuring the formation of H_2S from Cys (B). Activities are given as nanomoles (minutes x milligrams protein)$^{-1}$.

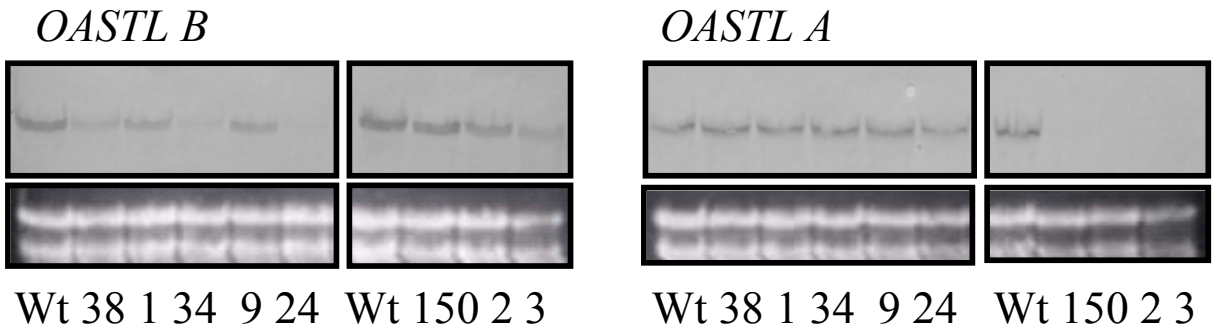


Fig. 3: Northern blot analysis of transgenic potato plants. The leaf material of five transgenic plants of the same line was combined and total RNA was extracted. A total of 15 µg RNA was loaded in each lane and blotted and probed with digoxigeninlabeled cDNAs of plastidic and cytosolic OASTL B and A, respectively. To prove equal loading of the extracted RNA, the ethidium bromide-stained RNA is shown at the bottom. The wild-type *OASTL* transcripts have a size of about 1.6 kb.

Measurements of OASTL protein content in transgenic potato lines and OASTL activity in a native gel

Protein extracts from leaves of the selected lines of OASTL antisense plants were subjected to protein analysis with an antiserum directed to OASTL protein (Fig. 4A). In the wild-type potato plants, the antiserum detected proteins with sizes of approximately 34 kD and 36 kD, respectively. This is in agreement with the predicted sizes of the cytosolic OASTL and the mature plastidial OASTL proteins, respectively, as deduced from the sequence analysis. Immunoblotting revealed a slight decrease in OASTL protein levels for the plastidial antisense plants (top band) but a significant decrease in the bottom band corresponding to cytosolic OASTL isoform in comparison to control plants. As judged by the immunoblot experiments, the level of OASTL protein was correlated only to the RNA blot for the cytosolic antisense plants, indicating that repression of *OASTL* transcript led to a reduced availability of the corresponding mRNA for translation. Using a second antibody directed against CAS, it was revealed that the content of this protein is not affected by decreases in either OASTL. Moreover, due to the similarity between OASTL and CAS at the protein level, a cross detection of the OASTL antiserum cannot be excluded and would explain the lower reduction level of OASTL in the plastidial antisense plants.

To further demonstrate that reductions in OASTL protein levels correlate with decreases in specific OASTL activity in leaves, protein extracts were separated on a native gel and analysed for OASTL activity (Fig. 4B). In agreement with the data obtained from RNA and

protein-blot analysis, the transgenic plants showed lower activities. The other activity seen can be assigned as CAS (Fig. 4B, bottom band).

Considering these results, we conclude that repression of the *OASTL* gene by antisense inhibition resulted in alterations of OASTL protein levels, which were in accordance to the corresponding protein quantities and enzyme activities in both transgenic lines.

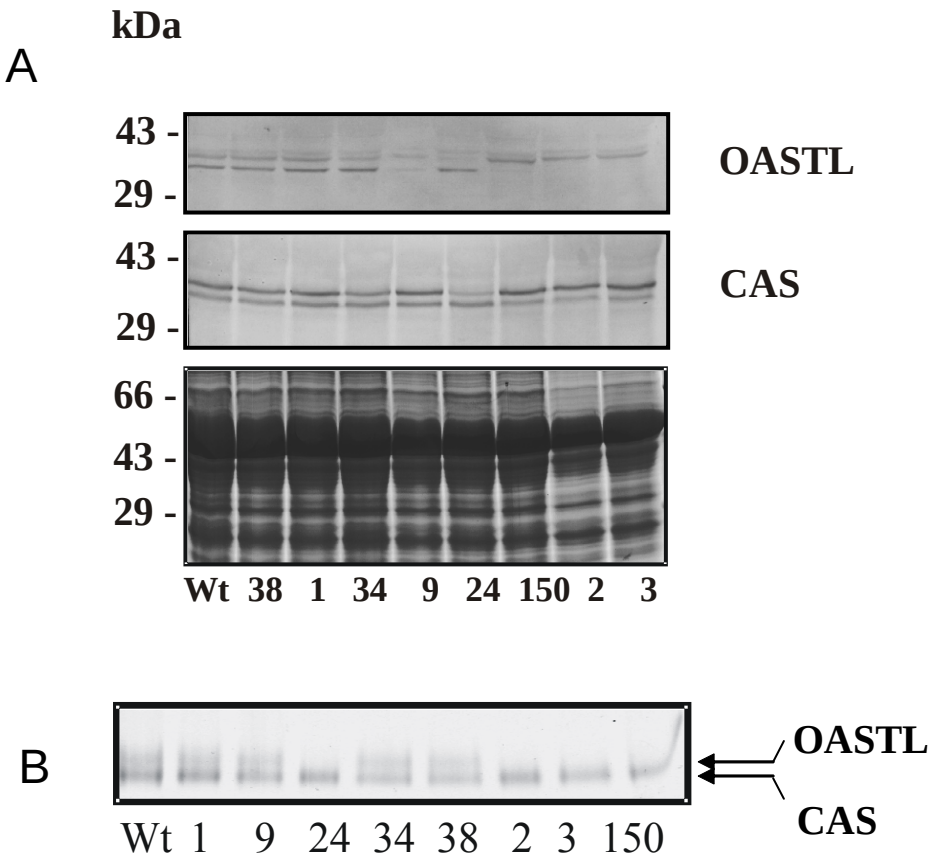


Fig. 4: Protein-blot analysis and activity staining of OASTL and CAS contents. A, For the protein blot-analysis, total protein extracts were prepared, separated on SDS-gels, and blotted onto nitrocellulose membranes. Monospecific antibodies recognizing the OASTL and CAS proteins, respectively, were used for the immunoreaction. The Coomassie-stained gel loaded with the same samples is shown in the lowest section to demonstrate loading of equal protein amounts. B, After native PAGE with 150 µg protein/lane, the gel was subjected to activity staining.

Effect of OASTL antisense inhibition on metabolite levels in source leaves

The effect of a decreased expression of the OASTL gene on the amounts of thiols and Asp-derived amino acid compounds was tested. Following the commonly held assumption that the de novo synthesis of amino acids in higher plants occurs in the chloroplasts, source leaf tissues were analysed for soluble metabolites using HPLC (Kreft et al. 2003) and gas chromatography-mass spectrometry (GC-MS)-based technology (Roessner et al. 2000, 2001). Analyzing the thiol levels revealed that in all transgenic plants, thiols such as Cys, γ-glutamylcysteine (GEC), and GSH increased significantly (Fig. 5, A–C). There is no direct correlation between the reduction of the OASTL activity and thiol content, but generally, these metabolites have higher levels in transgenic plants than in wild-type control plants. Furthermore, plants with higher Cys levels also have higher levels of GEC and GSH. For example, line 34 contained 1.7-fold more Cys, 2.0-fold more GEC, and 1.8-fold more GSH. Line 3 had comparable increases in Cys (1.9-fold), GEC (2.0- fold), and GSH (1.9-fold). It is important to note that almost all lines accumulated Ser (except line 1), the immediate precursor of OAS production (Fig. 5D). This is of importance because lines 38, 24, and 2 showed elevated but not significantly elevated levels of OAS (Fig. 5E). Since this intermediate is hardly detectable in leaves of wild-type plants, its degree of accumulation could not be determined in a practical manner. An increase of SAT protein was not detected (data not shown). Furthermore, although statistically not significant, all levels of amino acids of the Asp family were increased (Fig. 5, F–I), Met (Fig. 5F), which receives its sulfide moiety from Cys, especially benefits from the increased Cys levels and accumulated up to 2-fold. The increase might initiate internal pathway regulation that explains the increase of other amino acids such as Lys (Fig. 5G), Thr (Fig. 5H), and Ile (Fig. 5I). Other metabolites were not affected by the antisense approach (data not shown).

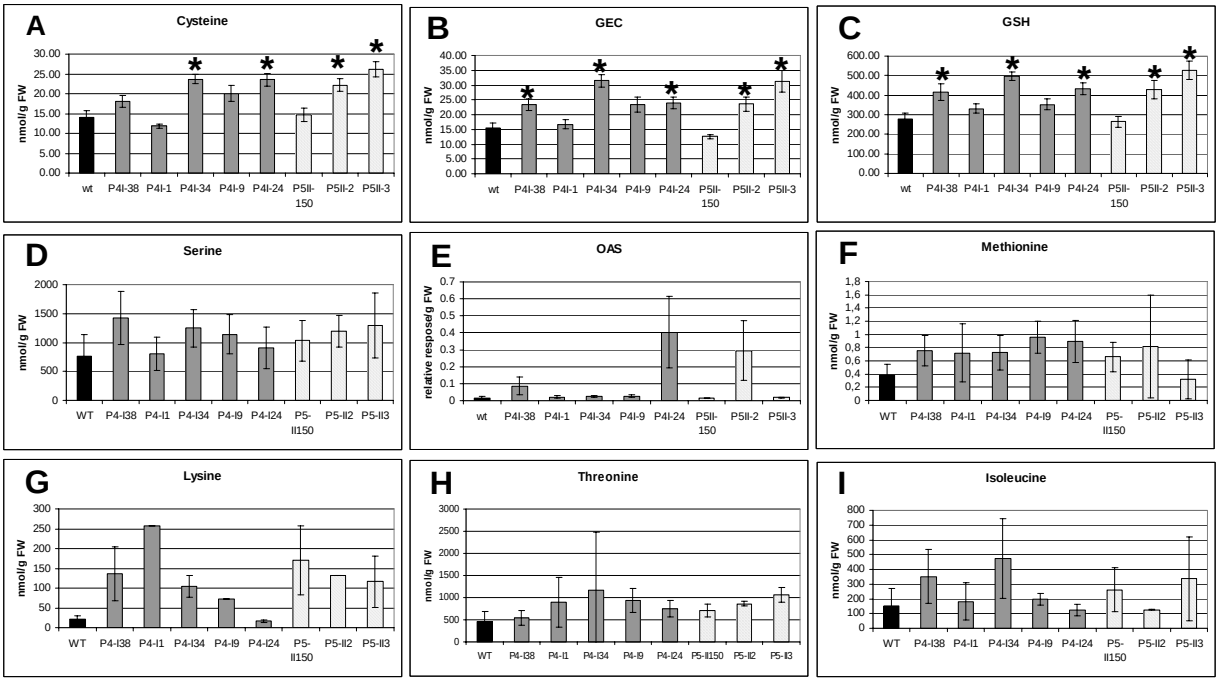


Fig. 5: Impact of reduced OASTL activity on the level of thiols, selected amino acids, and OAS. Metabolites were extracted from leaf tissues of 8-week-old plants. Cys (A), GEC (B), and GSH (C) were determined by HPLC analysis with monobrombimane derivatization and fluorescence detection. Amounts of thiols are given in nanomoles per gram fresh weight (FW) and represent mean values ± SD (n= 5). Differences between wild-type and transgenic plants analysed using Student's *t* test were statistically significant (asterisks, $P< 0.05$). Ser (D), Met (F), Lys (G), Thr (H), and Ile (I) were determined by HPLC analysis with *O*-phtaldialdehyde derivatization and fluorescence detection. Amounts of amino acids are given in nanomoles per gram FW and represent mean values ± SD (n= 5). E, OAS was measured from leaf material using GC-MS. Relative response is determined as ratio area metabolite/area internal standard. Data are presented as mean ± SD of five individual plants per line, one measurement per plant.

Discussion

Cys represents a key compound with several cellular functions being a proteinogenic amino acid, sulfur donor, or part of protective metabolites (Hesse et al. 2004). The multitude of implementations in the cellular network makes it necessary to regulate the synthesis and cellular homeostasis tightly. Cys synthesis can be regulated at several steps (Hesse et al. 2004). Most of our understanding on this particular step is based on studies of *E. coli* (Kredich et al. 1969) and later studies showing that related regulatory networks are present in higher plants (Hesse et al. 2004). Recent efforts have identified the SAT/OASTL complex as a suitable regulator of Cys synthesis. The current model states that SAT is active in the complex, while OASTL is inactive, resulting in OAS formation and disruption of the complex, followed by Cys synthesis (Hesse et al. 2004). On the other hand, excess OASTL in transgenic tobacco (*Nicotiana tabacum*) plants revealed a more tolerant phenotype when

exposed to oxidative stress but with a minimal increase in thiols under normal growth conditions (Noji et al. 2001; Youssefian et al. 2001; Sirko et al. 2004). In this paper, an antisense-mediated down-regulation of OASTL activity was used to misbalance the SAT/OASTL ratio to investigate the particular roles of OASTL isoforms in Cys formation and homeostasis. A set of transgenic plants was investigated expressing antisense RNA for the cytosolic and the plastidial isoform, respectively. Surprisingly, no effect on the growth habit of the transgenic lines could be observed, although OASTL activities were maximally reduced to 5% in crude extracts of transgenic plants. Generally, the reduction obtained with the cytosolic isoform was higher than for the plastidial one. An explanation for this result remains to be found. Constructs were made with the full-length cDNAs and the impact of antisense down-regulation cannot be predicted. However, this finding might indicate that the effectiveness of the cytosolic antisense RNA is higher than the plastidial RNA with its extension of the plastidial-targeting coding enzyme, sequence, even though they share 68.1% identity at the nucleic acid level. Another explanation could be that the molar ratio between cytosolic and plastidial OASTL activity is displaced in potato toward higher cytosolic activity as occurs in pea (*Pisum sativum*; Lunn et al. 1990). The down-regulation of OASTL activity is clearly a result of the antisense approach. RNA-blot analysis revealed a specific reduction in *OASTL* RNA levels of the antisensed isoform in transgenic plants corroborated by the enzyme activity measurements, protein-blot analysis, and the zymogram.

OASTL catalyses the incorporation of reduced sulfur into organic compounds. Modifying the enzyme activity at such a critical position in a pathway should therefore lead to alterations in metabolic turnover and affect levels of the respective biosynthetic end product. In this respect, changes in OASTL activity levels are likely to result in alterations of soluble Cys contents. Such a possibility has not been investigated in *Arabidopsis* or other plants. Overexpression of OASTL in *Arabidopsis* and tobacco has been shown to result in only moderate increases in Cys and GSH levels, though these increases led to augmented tolerance to oxidative stress or cadmium exposure (Harada et al. 2001; Noji et al. 2001; Youssefian et al. 2001; Sirko et al. 2004). Earlier studies revealed that OASTL is in excess (up to 400-fold) over the amount required to provide the overall flux of the pathway and is therefore not limiting to the rate of Cys synthesis in plants (Schmidt & Jäger 1992; Ruffet et al. 1995; Sirko et al. 2004). Taking this into consideration, one would expect that Cys synthesis would not be affected or even reduced by decreases in expression. It is surprising then that the reduction of OASTL activity and subsequent increases in the SAT/OASTL ratio in potato provoked a substantial and statistically significant increase in Cys levels (and in GEC and GSH) in a manner similar to

that seen in transgenic plants overexpressing SAT (Blaszczyk et al. 1999; Harms et al. 2000; Noji & Saito 2002; Wirtz & Hell 2003). Here, the SAT/OASTL ratio was elevated, resulting in higher thiol levels, which indicate that OAS is limiting. Taking these results together, one has to conclude that the formation of the SAT/OASTL complex might have two functions: the activation of SAT to form OAS and the protection of Cys homeostasis by binding OASTL, which is inactive and thus inhibiting the OASTL driven reverse reaction (Fig. 6).

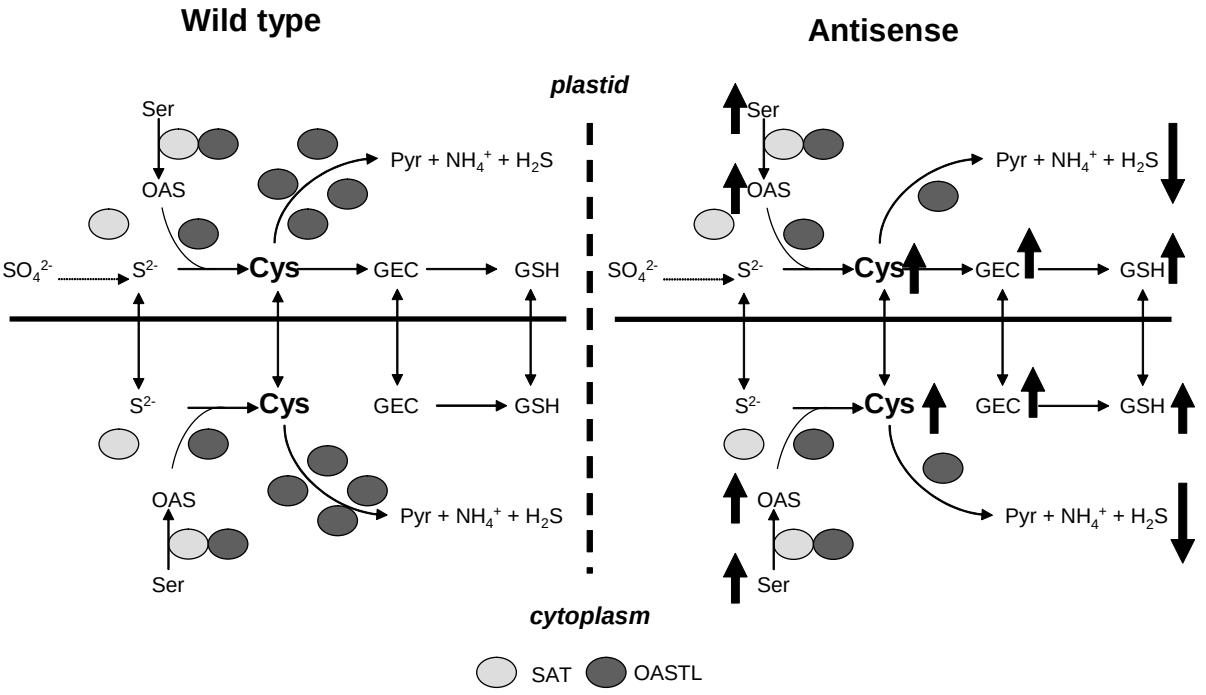


Fig. 6: Comparative model of Cys biosynthesis control in potato wild-type and transgenic plants. In wild-type plants, Cys homeostasis is controlled by the formation of the SAT/OASTL complex and the excess of free OASTL protein being able to catalyse the reverse reaction. In transgenic potato, however, the catabolic activity is reduced resulting in increased thiol levels independent from the down-regulated OASTL isoform. Precursor levels of Cys synthesis such as Ser and OAS increase in their amounts, too. Furthermore, OASTL isoforms are able to substitute each other by exchange of metabolites via the chloroplast membrane. Thus, in potato, the relative enzymatic excess of OASTL over SAT controls the Cys status. Pyr, Pyruvate; S^{2-}, sulfide; SO_4^{2-}, sulfate; SO_3^{2-}, sulfite; Ser, serine. Arrows indicate the increase and decrease, respectively, of metabolites.

Both the synthesis of OAS as Cys precursor and the reduced degrading activity of OASTL cause the increase in soluble thiols. This might also explain why SAT, as a more N-related is up-regulated by S deprivation (Takahashi et al. 1997; Hirai et al. 2003; Nikiforova et al. 2003). Nonetheless, the reduced capability to produce Cys apparently seems to generate a bottleneck in the Cys pathway, which is indicated by the accumulation of OAS and the precursor Ser. An increase in SAT protein was not observed. A plausible explanation for this finding could be the fact that the reduction in OASTL protein resulted in fewer binding partners for SAT, which causes the increase in Ser levels. If SAT is bound to OASTL, OAS can be synthesized but no longer used for Cys formation because of the reduction of free OASTL protein (Fig. 6). The increase in Cys levels has no effect on cellular sulfate levels (data not shown; Hesse et al. 1997) but does influence Met levels in transgenic plants. Virtually all the transgenic plants have higher Met levels than wild-type plants. Other amino acids of the Asp family are also increased by the manipulation. This can be explained by the internal regulation of the pathway (Hesse & Höfgen 2003). Increasing Met levels might result in more *S*-adenosylmethionine (not determined), which positively regulates Thr synthase activity. The investigated transgenic plants have increased Thr but also Ile levels. Remarkably, Lys levels are also increased, which cannot be explained by the current regulation model of the pathway. Moreover, a positive correlation between free Lys and Met could be observed for *Arabidopsis* plants modified in Lys metabolism (Zhu & Galili 2004). Other nonpathway-related amino acids or metabolites did not show significant alterations in leaves.

The increase in Cys and GSH levels in both types of antisense plants indicate that sulfur-related metabolites are exchanged between compartments, at least between chloroplasts and cytosol. Although the activity of the plastidial isoform is down-regulated, thiol levels were increased. It has to be assumed that sulfide, although very reactive, is able to cross the plastidial membrane, suggesting that both isoforms can substitute for each other (Fig. 6). Other compounds such as GEC must also cross organelle membranes, since GSH synthetase is localized in both cytosol and chloroplasts while GEC synthetase occurs only in plastids (Cobbett et al. 1998; The *Arabidopsis* Genome Initiative 2000; Noctor et al. 2002; Hartmann et al. 2003). Through this interorganelle transfer, the plant cell is able to keep Cys homeostasis constant, although it is questionable how the cells sense the Cys content in each compartment. In this context, it is of interest why OASTL protein is in such an excess over SAT. Apparently, from the obtained results, it can be assumed that the excess of OASTL is, under normal conditions, responsible for both de novo synthesis and breakdown of Cys to

keep Cys or reduced sulfur levels in balance (Fig. 6). The breakdown of Cys, the inverse reaction of OASTL (Burandt et al. 2002), results in pyruvate, ammonia, and H_2S production. The latter is volatile and could possibly be used as latent natural pathogen protection. A reduced H_2S-releasing capacity in the transgenic potato plants was observed; it would be interesting to investigate if these plants are less resistant to pathogen infections. An H_2S-releasing activity of true Cys desulfhydrase proteins is probably induced only in certain physiological states. There are a number of Cys desulfhydrase candidates in the *Arabidopsis* genome such as NifS-like proteins (Leon et al. 2002). However, the percentage of the turnover of Cys to H_2S catalysed by desulfhydrases and by the side reaction of OASTL proteins is not known.

The presented data provide evidence for a more complex regulation of Cys synthesis and homeostasis. The formation of the SAT/OASTL complex is likely part of the regulatory cascade. The obtained data indicate that other players are involved in the regulation of Cys biosynthesis in plants and that the current models require adjustments.

Chapter 3

Impact of elevated H_2S on metabolite levels, activity of enzymes and expression of genes involved in cysteine metabolism

The effects of elevated atmospheric hydrogen sulfide (H_2S) levels (0.25, 0.5, and 0.75 µl l^{-1}) have been investigated in a short-term exposure experiment (3–48 h) on the model plant *Arabidopsis thaliana* (L.) Heynh. in comparison to untreated control plants. The most pronounced effects of H_2S fumigation could be observed on the metabolite level: The contents of the thiols cysteine and glutathione were increased up to 20- and fourfold, respectively. A direct positive correlation of the thiol contents with the H_2S concentrations applied was observed. To elucidate the molecular basis for the increased thiol levels, enzyme activities, messenger RNA and protein steady-state levels of cysteine-synthesizing and degrading pathways have been determined. The enzyme activities of *O*-acetyl-L-serine(thiol)lyase (OAS-TL) (EC 4.2.99.8) and L-cysteine desulfhydrase (EC 4.4.1.–) proteins were not significantly higher at elevated H_2S levels in comparison to untreated control plants. 3-Mercaptopyruvate sulfurtransferase (EC 2.8.1.2) activity was slightly higher after the longest H_2S exposure times. Elevated H_2S levels of 0.25 and 0.5 µl l^{-1} had promoting effects on both mRNA and protein levels of cysteine-synthesizing and degrading enzymes whereas the highest H_2S concentrations caused lower levels of expression combined with mild symptoms of oxidative stress, as the consequence of its phytotoxicity. The differences in the expression of the three different *OAS-TL* isoforms (cytoplasmic, plastidic and mitochondrial) by H_2S were very small. Increasing concentrations of H_2S and longer exposure times to H_2S let to a reduction in the pool of *O*-acetyl- L-serine, the second precursor of cysteine, and *N*-acetyl-L-serine in the leaves and shoots, indicating a substrate depletion in agreement with the increased thiol levels.

Introduction

The impact of atmospheric hydrogen sulfide (H_2S) on plants is paradoxical. On the one hand, it may be utilized as a sulfur nutrient, and on the other hand, above a certain threshold level it may negatively affect plant growth and functioning (De Kok et al. 2002a). The predominant

natural sources of H_2S in terrestrial ecosystems are the biological decay of organic sulfur and the activity of dissimilatory sulfate-reducing bacteria (Bates et al. 1992; Beauchamp et al. 1984). Also plants contribute to biogenic H_2S emission with about 2–3 kg ha^{-1} and year (Schröder 1993). H_2S is supposed to be highly fungi-toxic (Beauchamp et al. 1984). Different field surveys have shown that a good sulfur supply can increase the resistance of agricultural crops against fungal pathogens (Schnug et al. 1995a). The mechanisms of sulfur-induced resistance (SIR) are, however, not yet known. Volatile sulfur compounds, such as H_2S, could play an important role in SIR (Bloem et al. 2004). On the other hand, the toxic H_2S concentrations for the respective plant species and the pathogens have not been determined in detail. To prove the hypothesis, data for H_2S toxicity on plants have to be completed, especially for the model plant *Arabidopsis thaliana* (L.) Heynh.

Sulfate is the primary source of sulfur for plants. Sulfate is taken up by the roots from the rhizosphere, reduced via several enzymatic steps, and finally *O*-acetyl-L-serine(thiol)lyase (OAS-TL) catalyses the biosynthesis of cysteine from H_2S and *O*-acetyl-L-serine (OAS) (for a review see (De Kok et al. 2002a; Leustek 2002)). It has been observed that foliarly deposited H_2S may be utilized as sulfur source for growth (Stuiver & De Kok 2001; Westerman et al. 2000; Westerman et al. 2001). The foliar uptake of H_2S is largely determined by the rate of assimilation of the deposited H_2S into cysteine by OAS-TL and the subsequent incorporation into other organic sulfur metabolites (De Kok et al. 1998; De Kok et al. 2002a; De Kok et al. 2002b; Van der Kooij & De Kok 1998). In *Brassica oleracea* L. (curly kale) at levels between 0.2 and 0.8 µl l^{-1} H_2S, which exceeded the plants sulfur requirement for growth, the adenosine-5′-phosphosulfate reductase enzyme activity decreased whereas the activities of ATP sulfurylase, serine acetyltransferase, and OAS-TL were not affected (Westerman et al. 2001). In *Arabidopsis* OAS-TL proteins are encoded by a small gene family (Jost et al. 2000). The nuclear-encoded OAS-TL isoforms are imported in different compartments of the cell: OAS-TL A remains in the cytoplasm (Hell et al. 1994), whereas OAS-TL B and OAS-TL C are imported into plastids and mitochondria, respectively (Hesse et al. 1999). The involvement of different OAS-TL isoforms in the assimilation of externally applied H_2S has not been investigated so far.

Under normal conditions, the cysteine content in plants is maintained at very low levels (Giovanelli et al. 1980) and it has been proposed that L- and/or D-cysteine desulfhydrase proteins play an active role therein (Schmidt & Jäger 1992). These enzymes catalyse the degradation of cysteine, resulting in the formation of H_2S, pyruvate and NH_4, or in elemental sulfur and alanine (Mihara & Esaki 2002). From *Azotobacter vinelandii* a protein, called NifS,

was shown to possess L-cysteine desulfhydrase activity and to be involved in Fe–S cluster biosynthesis (Zheng et al. 1993). NifS proteins are ubiquitous, homodimeric proteins which belong to the α-family of pyridoxal-5′-phosphate (PLP) depending enzymes. In cyanobacteria, a second group of proteins providing sulfur for Fe–S clusters *in vitro* was identified. These so called C-DES proteins possess L-cysteine/ cystine C-S-lyase activity (Leibrecht & Kessler 1997). Recently, NifS-like proteins were also identified in *Arabidopsis* localized in the mitochondrion (AtNFS1) and in the chloroplast (AtNFS2), respectively (Kushnir et al. 2001; Leon et al. 2002). Some sulfurtransferase proteins utilize a cysteine derivative, 3-mercaptopyruvate, as substrate and might therefore also be involved in the regulation of the cysteine pool (Papenbrock & Schmidt 2000).

So far the studies investigating the effects of atmospheric H_2S on the model plant *Arabidopsis* has been rather limited. Most experiments were focused on the biochemical analysis of several other plant species during H_2S fumigation. To be able to extend our experiments on the transcriptome and the proteome we have chosen *Arabidopsis*. A more complete picture of the transcript and protein levels, as well as enzyme activity and metabolite measurements might help to understand the regulatory mechanisms of the maintenance of the cysteine pool size and metabolism influenced by increased levels of atmospheric H_2S. The expression analysis of the OAS-TL isozymes during H_2S fumigation could clarify the role of different cellular compartments, the cytoplasm, mitochondria, and plastids, in assimilatory and dissimilatory sulfur metabolism.

Methods

Growth, treatment and harvest of plants

Seeds of *Arabidopsis*, ecotype C24, were originally obtained from the *Arabidopsis* stock center at the Ohio State University. Seeds were placed in Eppendorf cups and soaked in 100 µl water, and then placed in the refrigerator (4°C). After 1 week 100 µl of 0.1% agar solution was added to disperse the seeds. The seeds were sown on sterilized Lentse potground (Hortimea, Elst, The Netherlands) in 7 cm diameter pots with about eight seeds per pot. The substrate contained in 1 m^3:0.8 m^3 garden peat, 0.2 m^3 baltic peat, 7 kg potting soil and 1 kg pg-mix 12-14-24 (N-P-K) fertilizer (YARA, Oslo, Norway). This fertilizer contains 1.75 mol sulfur per kg. Calculated on the pot basis, at least 300 µmol sulfur were available in each pot with a volume of 170 cm^3. The pots were placed in a climate controlled room with a photoperiod of 16 h light at a photon fluence rate of 60 µmol* m^{-2} *s^{-1} (PAR 400–700 nm) supplied by Osram TL 31 and 21 in a ratio of 2:1 at a temperature of 23/21°C. After 2 weeks

the number of plants per pot was thinned to five per pot. Four weeks later the plants were transferred into 150-l stainless steel cylinders (diameter 0.6 m) with polycarbonate lids. The temperature was 21 ± 1°C and relative humidity of the air was 40%. The light intensity was 250 µmol* m^{-2} *s^{-1} (PAR 400–700 nm) with a Philips HPL(R)N (400 W) light. The air temperature was controlled by adjusting the cabinet wall temperature. The air exchange was 40 l min^{-1} and the air inside the cabinets was stirred continuously by a ventilator. Pressurized H_2S diluted with N_2 (1 ml l^{-1}) was injected into the incoming air stream and adjusted to the desired level by ASM electronic mass flow controllers (Bilthoven, The Netherlands). The watered plants were incubated for 0, 3, 6, 12, 24, and 48 h at 0, 0.25, 0.5, and 0.75 µl l^{-1} H_2S. The H_2S levels in the cabinets were controlled with an SO_2 analyser (model 9850) equipped with an H_2S converter (model 8770, Monitor Labs, Measurement Controls Corporation, Englewood, CO, USA). The plant parts above soil from 25 plants per treatment were harvested and directly frozen in liquid nitrogen. New plants were transferred to the cabinet after every harvest.

RNA extraction and Northern blot analysis

Total RNA was extracted essentially as described (Chomczynski & Sacchi 1987). RNA samples (between 15 and 25 µg dependent on the abundance of the respective mRNA) were separated on 1% denaturing agarose-formaldehyde gels. Equal loading was controlled by staining the gels with ethidium bromide. After RNA-transfer onto nylon membranes, filters were probed with digoxigeninlabeled cDNA probes obtained by PCR. The template clones for AtNFS1 (At5g65720), AtNFS2 (At1g08490), and D-CDes (At1g48420) were isolated in our laboratory from the λYES cDNA library (Elledge et al. 1991) or via reverse transcriptase PCR. To amplify the respective probes the following sequence specific primers were used: For AtNFS1 primer 22 (5′-CTG GAT CCG CGT CTA AGG TAA TCT-3′) and primer 23 (5′-CCC TGC AGT CAG TGT TGA GAC CAT T-3′), for AtNFS2 primer 64 (5′-CGG TAC CTT TGA AAG AGT TGA AGA AGC TCA C-3′) and primer 69 (5′-CGG ATC CGA AGGTGT GGCTAT GAAA-3′), and for D-CDes primer 102 (5′-CGG ATC CAG AGG ACG AAG CTT GAC A-3′) and primer 103 (5′-CTG CAG GAA CAT TTT CCC AAC ACC-3′). The cDNA for AtP1 (At5g16970) was obtained from Dr. S. Kushnir, Ghent, Belgium, and was amplified using primer 278 (5′-GGA TCCACG GCGACGAACAA-3′) and primer 279 (5′-CTG CAG CGA GCA ACA ACA ACA-3′). The PCR conditions for labeling the cDNAs from *Arabidopsis* encoding OAS-TL A–C are described in (Burandt et al., 2001), for CAS in (Meyer et al. 2003), and for AtStr1 in (Papenbrock & Schmidt 2000). Colorimetric or

chemiluminescent detection methods with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) or with CDPStar (Roche, Mannheim, Germany) as substrates for alkaline phosphatase were applied.

SDS-PAGE and Western blot analysis

For the determination of steady-state protein levels in plants, 100 mg plant material was ground with a mortar and pestle in liquid nitrogen to a fine powder. Five hundred microliters sample buffer (56 mM Na_2CO_3, 56 mM dithiothreitol (DTT), 2% SDS, 12% sucrose, 2 mM EDTA) was added, samples were heated at 95°C for 15 min, and cell debris was removed by centrifugation. Ten micrograms of total protein was subjected to denaturing SDS gel electrophoresis according to (Laemmli 1970) and blotted (Sambrook et al. 1989).A colorimetric detection method using NBT and BCIP as substrates for alkaline phosphatase was applied. Monospecific antibodies produced against the purified CAS and OAS-TL proteins from spinach, respectively (Hatzfeld et al. 2000), and antibodies produced against the recombinant *Arabidopsis* D-CDes protein were used for immunodetection.

Enzyme activity measurements

Plant material was ground with a mortar and pestle in liquid nitrogen and the soluble proteins were extracted by adding 1 ml 20 mMTris/HCl, pH 8.0, to 100 mg plant material. After centrifugation the protein content of the supernatantwas adjusted to 100 µg ml^{-1} to obtain equal amounts of protein in each assay sample.

L-cysteine desulfhydrase activity was measured by the release of H_2S from L-cysteine in the presence of DTT. The assay contained in a total volume of 1 ml: 0.8 mM L-cysteine, 2.5 mM DTT, 100 mM Tris/HCl, pH 9.0, and 10 µg protein solution. The reaction was initiated by the addition of L-cysteine; after incubation for 15 min at 37°C the reaction was terminated by adding 100 µl of 30 mM $FeCl_3$ dissolved in 1.2 N HCl and 100 µl 20 mM *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride dissolved in 7.2 N HCl (Siegel 1965). The formation of methylene blue was determined at 670 nm in microtiter plates using a microtiter plate reader (Fluostar Optima, BMG Labtechnologies, Offenburg, Germany). Solutions with different concentrations of Na_2S were prepared, treated in the same way as the assay samples, and were used for the quantification of enzymatically formed H_2S. D-cysteine desulfhydrase activity was determined in the same way with following modifications: D-cysteine instead of L-cysteine was used, the pH of the Tris/HCl buffer was 8.0. The OAS-TL activities were determined as described using 50 µg protein extract (Burandt et al. 2001c). 3-

Mercaptopyruvate sulfurtransferase activity was measured as described using 50 µg protein extract (Meyer et al. 2003).

Determination of metabolites

Fifty mg of plant material ground in liquid nitrogen was extracted with 500 µl 0.1 N HCl. The samples were centrifuged two times for 5 min at 16,060 × *g* and 4°C. For derivatization the plant extracts were prepared in the following way: 220 µl H_2O, 20 µl 1Mtris/HCl, pH 8.0, and 10 µl of 10mM DTT were mixed with 10 µl plant extracts and neutralized with 10 µl 0.08 N NaOH. The sulfhydryl groups were derivatized in the dark with 20 µl 10 mM monobromobimane (Sigma, Taufkirchen, Germany) dissolved in acetronitrile as described by (Hell & Bergmann 1990). The derivatization was stopped with 710 µl 5% acetic acid. Separation, detection, and quantification of the fluorescent adducts was achieved by a reversed phase column (Nova-Pak C_{18}, 4 µm, 3.9 × 150 mm, Waters, Milford, MA, USA) and an HPLC system equipped with a fluorescence detector (Knauer, Berlin, Germany). Thiols were eluted with a gradient of solvent B (100% methanol) in solvent A (0.1 M potassium acetate buffer, pH 5.5) at a flow of 0.5 ml min^{-1} as follows: 0 to 18.5% over 4 min, 18.5% for 5 min, 18.5–100% over 2 min, and 100% solvent B for 13 min. Column eluent was monitored by fluorescence detection (λ_{ex} 380/λ_{em} 480). The thiols cysteine and glutathione were quantified using authentic standards. In the same plant material the precursor of cysteine, OAS, NAS, and several other metabolites were determined by gas chromatography/mass spectrometry (GC–MS)-based technology according to (Nikiforova et al. 2003; Roessner et al. 2001).

Miscellaneous and statistical evaluation

Protein estimation was performed according to (Bradford 1976) using bovine serum albumin as a standard. The H_2S fumigation experiments were repeated three times with similar results (biological replication). For the analyses presented in this paper only the results of experiment three were included. Each type of analysis was done at least in triplicate with the same frozen plant material (technical replication). In addition each data point of the thiol and the enzyme activity determinations were obtained by duplicates. Representative Northern and Western blots out of three replicates are shown.

Results

Influence of H_2S fumigation on the phenotype of the Arabidopsis *plants*

The plants were carefully checked for any visible symptoms caused by H_2S fumigation. At H_2S concentrations of 0.25 and 0.5 µl l^{-1} the fumigated plants did not develop any noticeable phenotype. After 12 h of fumigation with the highest H_2S concentration of 0.75 µl l^{-1} the plants developed symptoms of turgor loss. The symptoms became more evident after longer treatment for 24 and 48 h, and some leaves showed small necrotic lesions.

Impact of elevated H_2S on thiol levels

The contents of cysteine as well as of the tripeptide glutathione were significantly increased in almost all *Arabidopsis* plants fumigated with H_2S (Fig. 1). The levels of cysteine and glutathione increased with increasing H_2S concentration and with increasing fumigation time. After 48 h of H_2S treatment the thiol contents slightly decreased. Obviously glutathione was immediately synthesized from increased amounts of cysteine. The highest increase in the cysteine content was measured after exposure for 24 h at the highest fumigation concentration of 0.75 µl l^{-1} H_2S. The increase of cysteine in comparison to the respective control was about 20-fold. The highest amounts of glutathione were determined in the same plant samples (about fourfold higher than the respective control). The results indicated a direct correlation of H_2S concentration and thiol levels. Either the H_2S is directly assimilated into cysteine and subsequently to glutathione or H_2S has a regulatory impact on the thiol contents.

Influences of H_2S on enzyme activity

To understand the basis for the significantly increased thiol levels, activities of cysteine-synthesizing and degrading proteins were determined (Fig. 2). The diagrams are divided into four blocks, one for the control without H_2S treatment (left), and one for each treatment from 0.25 up to 0.75 µl l^{-1} H_2S. The fumigation with H_2S resulted in a small increase of L-cysteine desulfhydrase activity in the plants fumigated for 12 h (Fig. 2A). The highest activity was measured in plants fumigated with a concentration of 0.5 µl l^{-1} H_2S for 12 h. After fumigation for 24 and 48 h the activity decreased slightly, but never below the levels in the control plants. The specific D-cysteine desulfhydrase activity determined in the same plant material did not change in H_2S-treated plants in comparison to the control plants (data not shown).

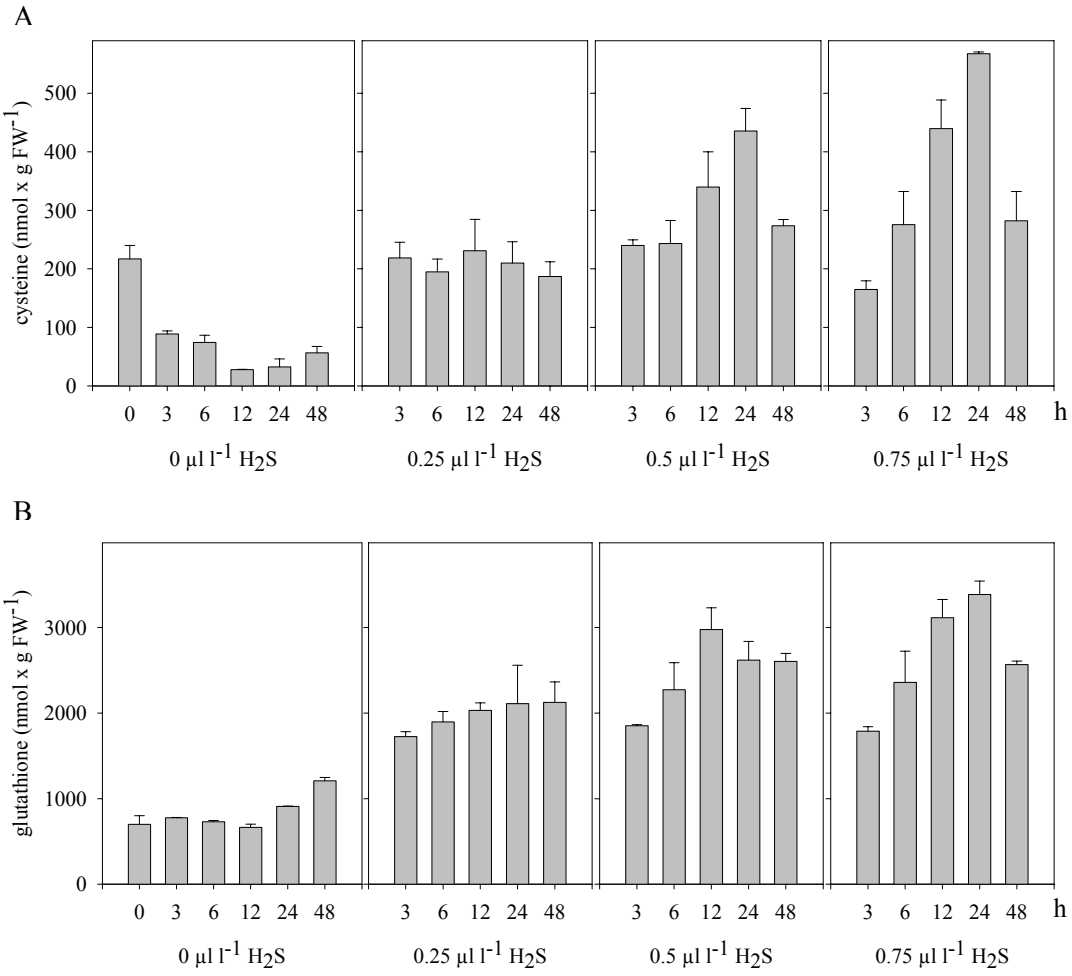


Fig. 1: The impact of H_2S fumigation on the thiol contents in *Arabidopsis* shoots. Four-week-old *Arabidopsis* plants were fumigated with different concentrations of H_2S (0, 0.25, 0.5, and 0.75 µl l^{-1}) for 3, 6, 12, 24, and 48 h under defined conditions. Shortly before beginning of the H_2S treatment control plants were harvested (indicated as 0 h-control). After the respective time points five pots with five *Arabidopsis* plants each were removed, the leaf material was cut, and immediately frozen in liquid nitrogen. Thiols were extracted with HCl from mortared plant material, labeled with monobromominane, and subjected to HPLC analysis using authentic standards for quantification. (A) Cysteine and (B) glutathione contents. Data represent the mean of three independent measurements (± S.D.).

The levels of OAS-TL activity also showed a maximum after 12 h of H_2S fumigation, almost independently on the respective H_2S concentration (Fig. 2B). In the control plants the activity remained equal during the experiment with the exception of the 0 h-control which had a significantly higher activity than the other plants. The sulfurtransferase activity catalyzing the degradation of the cysteine derivative 3-mercaptopyruvate increased with increasing H_2S fumigation time and concentration (Fig. 2C).

Steady-state protein levels

Next the protein steady-state levels of selected cysteine synthesizing and degrading proteins were investigated. For the Western blot analysis antibodies directed against β-cyano-L-alanine synthase (CAS), OAS-TL, and D-cysteine desulfhydrase (D-CDes) were used (Fig. 3). For a better comparison the 0 h-control was loaded two times, in lane one of block one and in lane one of block three. The CAS antibody recognized two bands in a range of 37–40 kDa. The intensity of the bands remained almost unchanged, except for a longer fumigation at the highest H_2S concentration. The antibody against OAS-TL detected one band at about 35 kDa. The intensity decreased after 12 h fumigation with 0.5 and 0.75 µl l^{-1} H_2S. The D-CDes antibody recognized one single band of about 42 kDa corresponding to the size of the mature D-CDes protein (Riemenschneider et al. 2005a). In the controls and in the plants fumigated with 0.25 µl $l^{-1}$$H_2S$ the steady-state protein levels remained almost constant. The higher H_2S concentrations influenced the steady-state D-CDes protein levels in different ways: At 0.5 µl l^{-1} H_2S the protein levels increased after longer exposure whereas at 0.75 µl l^{-1} H_2S the protein levels decreased.

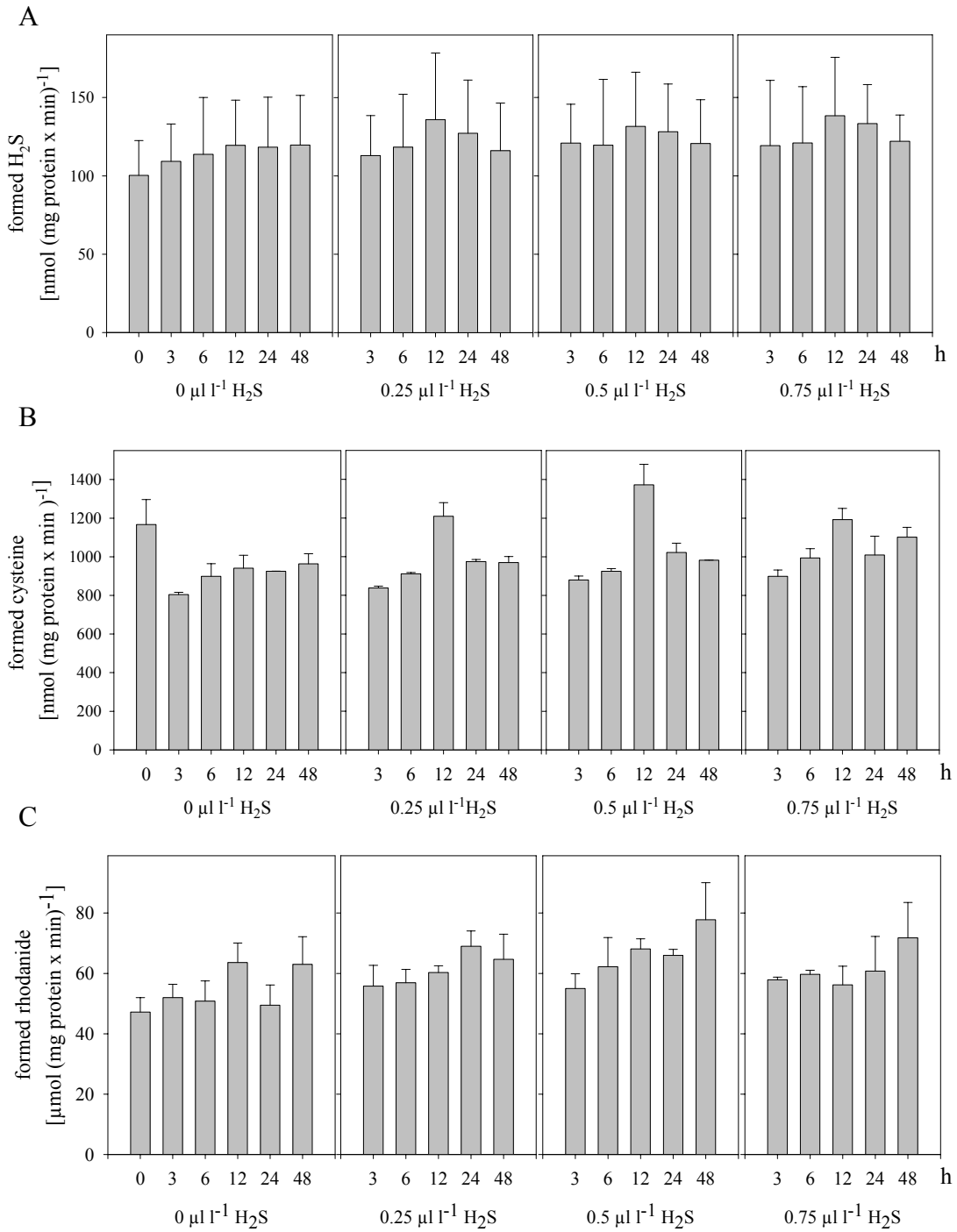


Fig. 2: Determination of enzyme activities. Soluble protein extracts were prepared from the same plant material as described in Fig. 1. (A) The graphs show the L-cysteine desulfhydrase activity measured as the amount of H_2S formed enzymatically. The H_2S was quantified with the help of a Na_2S standard curve. (B) The OAS-TL activity was determined by measuring the amount of enzymatically formed cysteine.A L-cysteine standard curve was used for quantification. (C) The sulfurtransferase activity was performed by using 3-mercaptopyruvate as sulfur donor and cyanide as sulfur acceptor. The formation of thiocyanate was quantified. All data represent the mean of three independent measurements (± S.D.).

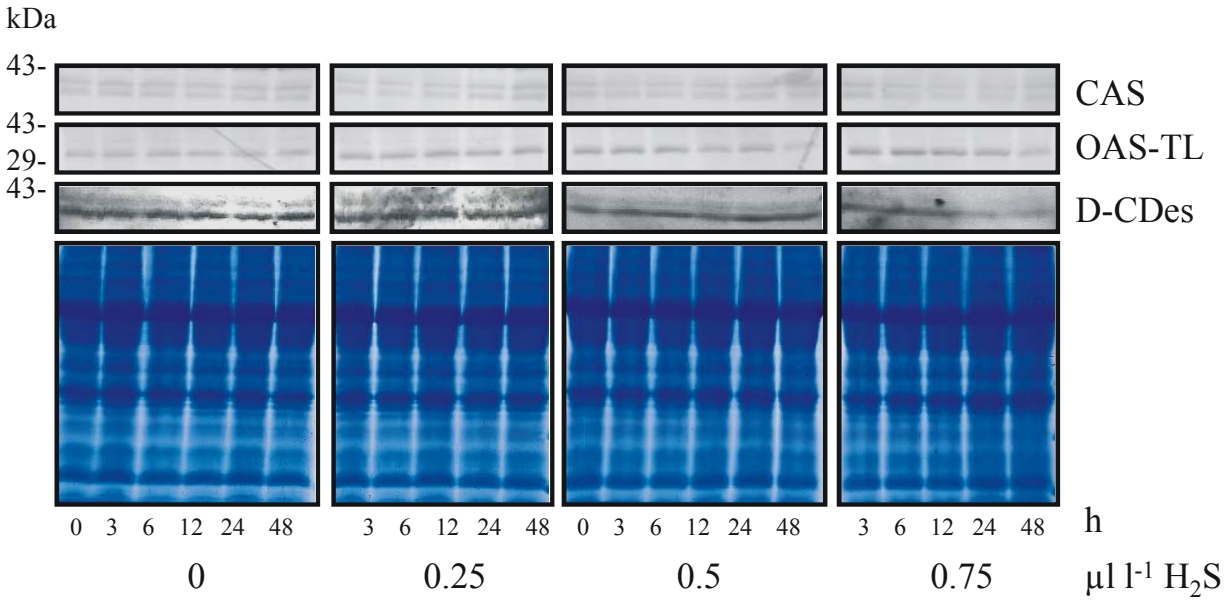


Fig. 3: Western blot analysis. The same plant material as described in Fig. 1 was subjected to Western blot analysis using antibodies directed against the CAS, OAS-TL, and D-CDes proteins. The Coomassie-stained gel loaded with the same samples used for the Western blots is shown in the lowest panel to demonstrate equal protein loading in each lane. Representative Western blots out of three replicates are shown.

Northern blot analysis of genes involved in sulfur metabolism

The expression pattern during H_2S fumigation of eight different cDNAs encoding proteins involved in the synthesis and/or in the degradation of cysteine or cysteine derivatives (three OAS-TL isoforms, CAS, AtNFS1, AtNFS2, AtStr1 [3-mercaptopyruvate sulfurtransferase 1], and D-CDes) were analysed (Fig. 4). The mRNA levels of the three OAS-TL isoforms A-C remained relatively constant. Their mRNA levels increased slightly with increasing H_2S concentrations and decreased at the highest concentration of 0.75 µl l^{-1} H_2S applied for more than 24 h. The highest mRNA level of *OAS-TL B* could be detected after a fumigation concentration of 0.25 µl l^{-1} H_2S. The *CAS* mRNA levels were slightly increased after fumigation for 24 or 48 h, however, a slight increase of *CAS* expression could also be observed in the 24- and 48 h-controls. The *AtNFS1* mRNA encoding a mitochondrial L-cysteine desulfhydrase (Kushnir et al. 2001) was down-regulated by the higher H_2S fumigation concentrations of 0.5 and 0.75 µl l^{-1}. Also the mRNA levels for a plastidic L-cysteine desulfhydrase, *AtNFS2*, were slightly down-regulated by higher H_2S concentrations. The mRNA levels of D-CDes encoding a D-cysteine desulfhydrase were decreased by fumigation with 0.5 and 0.75 µl l^{-1} H_2S. The *AtStr1* mRNA levels were unchanged. In summary, the mRNA levels for the cysteine-synthesizing OAS-TL proteins did not change. The mRNA for the cysteine desulfhydrase proteins were down-regulated.

Does the fumigation with H_2S cause oxidative stress in* Arabidopsis *plants?

The *AtP1* gene from *Arabidopsis* belongs to the plant zetacrystallin family containing two other genes and is homologous to NADPH oxidoreductases sequences. It was observed that the *AtP1* steady-state mRNAs accumulated rapidly in *Arabidopsis* plants under various oxidative stress conditions, such as treatment with paraquat, t-butylhydroperoxide, diamide, and menadione (Babiychuk et al. 1995). Recently, it was shown that the AtP1 protein functions by scavenging the highly toxic, lipid peroxide-derived α, β-unsaturated aldehydes and plays a major role in defence against oxidative stress (Mano et al. 2002). The *AtP1*mRNA levels increased after fumigation with higher concentrations of H_2S independently from the duration of the treatment (Fig. 4). The highest levels were detected at 0.75 µl l^{-1} H_2S. There might be a decrease of expression after 12 h at all H_2S concentration levels. To exclude effects of a diurnal rhythm, the *AtP1* expression was followed during a 12 h light/12 h dark cycle, however, any differences in the *AtP1*mRNA level could not be observed (data not shown). In summary, the plants fumigated with the highest H_2S concentration of 0.75 µl l^{-1}, particularly for a longer exposure time, did suffer under mild oxidative stress indicated by slightly increased expression levels of *AtP1*.

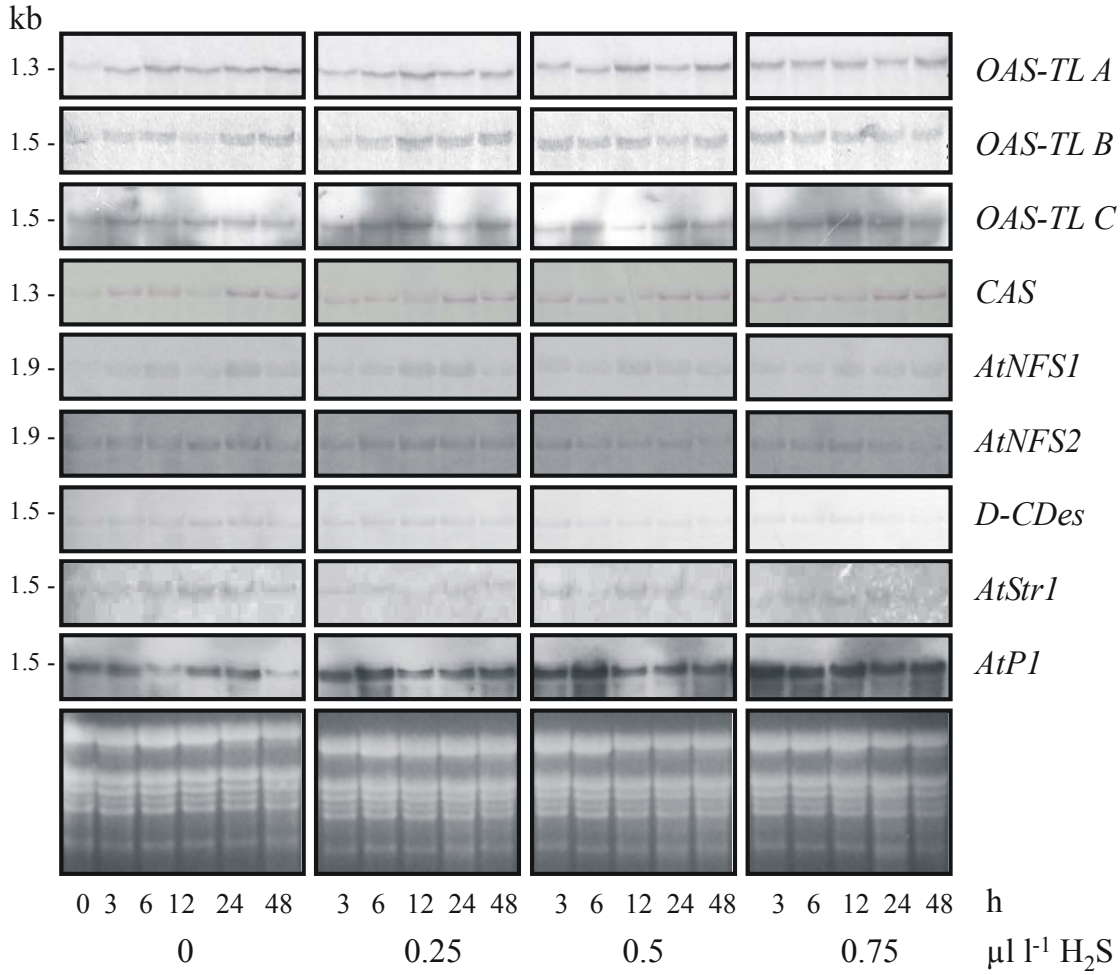


Fig. 4: Northern blot analysis. The same plant material as described in Fig. 1 was subjected to Northern blot analysis. Total RNA was probed with digoxigenin-labeled cDNAs coding for three OAS-TL isoforms, A–C, for CAS, AtNFS1, AtNFS2, D-CDes, AtStr1, and AtP1. To control equal RNA loading the agarose gels stained with ethidium bromide are shown at the bottom. Representative Northern blots out of three replicates are shown.

Impact on thiol precursors and other metabolites

As shown in Fig. 1 the contents of cysteine and glutathione were significantly increased in almost all *Arabidopsis* plants fumigated with H_2S. To investigate the effects of elevated H_2S concentrations on the contents of the second precursor of cysteine, OAS, and also *N*-acetyl-L-serine (NAS) were determined (Fig. 5). The sum of OAS and NAS contents were reduced with increasing H_2S concentration and duration of treatment. The OAS/NAS contents decreased by a factor of 1.4 for 0.25 µl l^{-1}, by a factor of 5 for 0.5 µl l^{-1} and by a factor of 15 for 0.75 µl l^{-1} H_2S. The results could be explained by substrate exhaustion corresponding to a higher cysteine and glutathione production. The differences in the OAS concentrations among the control plants were relatively large. Maybe the OAS and/or NAS contents cycle in a diurnal rhythm. The availability of free amino groups of OAS is pH-dependent and decrease

between pH 7.0 and 8.0 drastically (about 75%) by conversion of OAS to NAS via a cyclic intermediate. The ratios of OAS to NAS contents were calculated, however, there was no specific pattern observed. There is only a small pH window for optimal *in vivo* OAS-TL enzyme activity. In this experiment the total amounts of OAS and NAS in cell extracts were determined; therefore pH differences and thus concentration differences between the compartments could not be taken into account.

The contents of seven amino acids and related compounds have been determined additionally. In summary, the contents of most of the compounds investigated decreased with increasing H_2S concentration. Two remarkable exceptions should be mentioned: Isoleucine levels were strongly enhanced by longer exposure at all three H_2S levels in comparison to the controls. The strongest effect was observed at the highest H_2S concentration (up to seven times in comparison to the respective control). Tryptophan levels were only slightly increased, but in the same way as isoleucine (data not shown). Allantoin levels showed a similar pattern as was described for OAS with the exception of the controls which did not reveal any changes (data not shown). Fumigation with H_2S did not only affect sulfur-containing compounds or direct precursors but also the metabolite levels in other pathways.

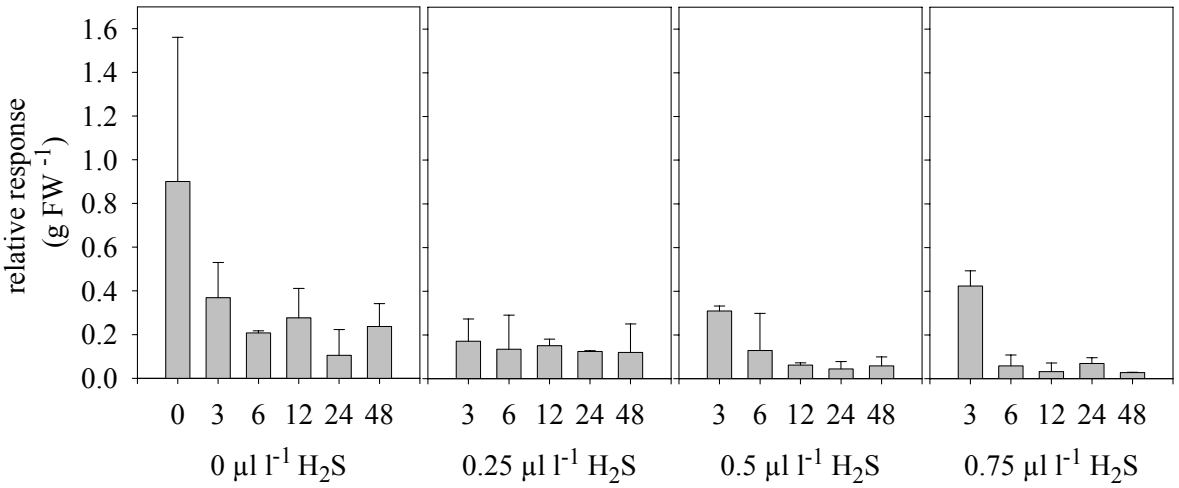


Fig. 5: Determination of OAS and NAS in H_2S fumigated *Arabidopsis* plants. OAS and NAS contents were determined by GC–MS in the same plant material as described in Fig. 1. The sum of both metabolites is shown. Data represent the mean of three independent measurements.

Discussion

In Arabidopsis *the levels of cysteine and glutathione were significantly increased by fumigation with H_2S*

The aim of this short-term H_2S exposure experiment was to determine the influence of H_2S on different cellular levels: on the metabolite concentrations and enzyme activities as well as on protein and gene expression. Additionally the range of H_2S concentrations, which are either beneficial or toxic for *Arabidopsis* plants, were analysed in detail for the first time. The *Arabidopsis* plants used for this investigation were supplied with relatively high amounts of sulfur. The *Arabidopsis* plants used for this investigation were supplied with relatively high amounts of sulfur. Per pot 300 µmol sulfur were available which is sufficient for growth as demonstrated in experiments done previously. For instance if *Brassica* plants were grown in 25% Hoagland solution and if their performance was compared with plants grown on the same substrate used for this experiment, the shoots hardly differed in growth, sulfate and total sulfur content (pers. communication Luit J. De Kok, Haren, The Netherlands). In general, H_2S exposure of plants results in a slight overload of the plant sulfur supply, which is illustrated by an increased size and change in composition of the thiol pool in the shoots (De Kok et al. 2002a; De Kok et al. 2002b). Likewise in *Arabidopsis* shoots there was a significant increase in cysteine and glutathione levels upon H_2S fumigation. The amounts of cysteine in theH_2S-exposed plants could be directly correlated with increasing H_2S concentrations and with the duration of the treatment, only after 48 h the cysteine levels slightly decreased. The same observations were true for glutathione. However, the absolute maximum increase in the glutathione levels was only about one fifth of the increased cysteine levels. However, the basic glutathione amount is about 800 nmol g^{-1} fresh weight, whereas the cysteine amount is about 100 nmol g^{-1} fresh weight in untreated plants. The physiological background of the changes in the composition of the thiol pool upon H_2S exposure is still unresolved and the subcellular site of metabolism of the deposited H_2S is still unknown. OAS-TL, the enzyme directly responsible for the fixation of atmospheric H_2S into cysteine, is present in both chloroplasts and cytosol, and in *Arabidopsis* also in the mitochondria. The increase in both size and altered composition of the thiol pool upon H_2S exposure could be due to an enhanced synthesis of cysteine in the cytosol, and in *Arabidopsis* also in the mitochondria, beyond the existing systems of feedback control of sulfate reduction and assimilation in the chloroplast (De Kok et al. 2002b). One has to keep in mind that the thiol fraction comprises only a small portion (about 2%) of the organic reduced sulfur fraction and the major proportion of the metabolized H_2S ends up in the proteins (Westerman et al. 2001).

The impact of elevated H_2S levels on activities of cysteine-synthesizing and degrading enzymes was limited

What are the possible consequences of a substantial increase in cysteine levels for activity and expression of the enzymes involved in cysteine metabolism? Both the activity of cysteine-synthesizing and degrading enzymes was not significantly influenced, with the exception of the slight increase in activity of 3-mercaptopyruvate sulfurtransferase. Also in previous long-term experiments of H_2S exposure for up to 8 d it was shown that the OAS-TL activity in *B. oleracea* was not the rate-limiting step in the H_2S uptake by shoots, even at sulfate deprivation (Stuiver & De Kok 2001). Also in *Arabidopsis* plants, a longer fumigation with H_2S for up to 7 d did not cause significant changes in the OAS-TL and L-cysteine desulfhydrase activities (data not shown).

One has to keep in mind that the conditions for *in vitro* enzyme activity measurements are optimised to measure maximum enzyme activities. However, it was shown that transgenic OAS-TL sense tobacco plants possessing two to three times higher OAS-TL activities were resistant to 100 $\mu l\, l^{-1}$ H_2S applied for 2 and 24 h. Only after 6 d of this extremely high H_2S concentration also the sense plants showed necrotic lesions comparable to the lesions on wild -type plants already visible after 2 h of H_2S fumigation (Youssefian et al. 1993). The finding that the catalytic capacity of OAS-TL exceeds sulfur assimilation needs by several 100-fold (Schmidt & Jäger 1992) may well be indicative for the direct role of this enzyme in the use and/or the detoxification of atmospheric H_2S without enhancing the overall enzyme activity by activation of enzyme molecules or by increasing the number of enzyme molecules. The tendency of OAS to decrease after exposure of *Arabidopsis* plants to H_2S supports this scenario (Fig. 5). OAS is synthesized by serine acetyltransferase (SAT). There is biochemical and molecular evidence that in plants, SAT and OAS-TL, are associated in a multi-enzyme complex called cysteine synthase, first described in *Salmonella typhimurium* and later in *Arabidopsis* (Kredich et al. 1969; Wirtz et al. 2001). The current model of cysteine formation proposes that in the formed complex of OAS-TL and SAT, OAS-TL is virtually inactive, but causes the stabilization of SAT while SAT is only active when bound in the complex. OAS formed in the complex now decreases the binding affinity of both enzymes and OAS-TL is released to convert OAS to cysteine. The dissociation of the complex serves to control OAS synthesis. However, because the concentration of OAS-TL is far in excess of SAT, the free OAS-TL is responsible for the production of cysteine (Hell et al. 2002).

Cysteine desulfhydrases might also catalyse the synthesis of cysteine as was shown for *Spinacia oleracea* L. extracts (Poortinga et al. 1997). One could assume that cysteine desulfhydrase proteins might contribute to the high cysteine contents in H_2S fumigated plants while using and/or detoxifying as much H_2S as possible. The physiological significance of this backward reaction is not known. In this study only the forward reaction has been determined.

In previous experiments enzyme activities have been determined every 4 h in *Arabidopsis* plants grown in a 12 h light/12 h dark cycle. The highest activities of OAS-TL, and L-cysteine and D-cysteine desulfhydrase were observed at the end of the light cycle 9 h after the onset of light (Burandt et al. 2001c; Riemenschneider et al. 2005a). This diurnal rhythm in enzyme activities might explain the maximum of activity after 12 h of H_2S fumigation surpassing the effects of H_2S. However, in the control plants this maximum could not be observed.

It was speculated that total amino acid pools might not reflect the actual L-amino acid concentration; the transformation into D-amino acids might constitute a form of compartmentation of amino acid concentration without a special compartment (Schmidt 1982). D-Cysteine desulfhydrase activity might control the total cysteine pool. Recently, a D-CDes cDNA has been isolated from *Arabidopsis*; its translation product showed D-cysteine desulfhydrase activity (Riemenschneider et al. 2005a). However, fumigation with H_2S had no effect on the D-cysteine desulfhydrase enzyme activity in *Arabidopsis* crude extracts. Up to now the pool sizes of D- and L-cysteine in *Arabidopsis* plants are not known, therefore the hypothesis can not be confirmed.

Short-term exposure of *B. oleracea* to H_2S resulted in a decrease in the activity of adenosine-5′-phosphosulfate reductase in the shoot (Westerman et al. 2001) indicating again a regulatory role of this key enzyme in sulfate assimilation on an early step in the pathway. However, a signal molecule was not identified so far. One could speculate, among other candidates, about H_2S itself involved in signaling. It would be interesting to follow also the expression of adenosine-5′-phosphosulfate reductase during H_2S fumigation to differentiate between a deactivation of the adenosine-5′-phosphosulfate reductase enzyme activity and a down-regulation on the transcriptional level by elevated H_2S concentrations.

In addition to glutathione biosynthesis cysteine might be derivatized leading to other pathways in sulfur metabolism. One example is the conversion of cysteine to 3-mercaptopyruvate as substrate for sulfurtransferases (Papenbrock & Schmidt 2000).An increase in sulfurtransferase activity was observed in dependency on the H_2S concentration. The highest activities were measured after longer exposure to H_2S when the cysteine levels

were already declining (Fig. 2C). The results might indicate that enzymes such as sulfurtransferases contribute to the detoxification of higher cysteine levels. In future experiments it would be interesting to investigate more putative catabolic pathways and their products to follow the destiny of synthesized cysteine.

Expression analysis and indication of oxidative stress

Atmospheric H_2S is phytotoxic and growth of susceptible species may already be negatively reduced upon prolonged exposure to atmospheric levels of $\geq$0.03 µl l^{-1}, whereas visible injury may occur at levels $\geq$0.3 µl l^{-1} (De Kok et al. 1998; De Kok et al. 2002b). The increased expression of *AtP1* at the highest H_2S concentration of 0.75 µl l^{-1} and the small necrotic lesions on the leaves indicated the occurrence of oxidative stress in *Arabidopsis* plants, which might be the consequence of the toxic effects of H_2S and a severely disturbed metabolism.

The physiological basis for toxicity of H_2S is still largely unclear. H_2S is very reactive and similar to cyanide it complexes with high affinity to metallo groups in proteins; this reaction is likely the primary biochemical basis for the phytotoxicity of H_2S (Beauchamp et al. 1984; De Kok et al. 1998; De Kok et al. 2002b). Many aquatic animal species can survive H_2S exposure to some extent through oxidation of the H_2S, which results mainly in thiosulfate (Grieshaber & Völkel 1998). However, in this experiment other sulfur-containing metabolites have not been determined. In the plant cell the biochemical mechanism of H_2S toxicity may involve inhibition of mitochondrial electron transport (Beauchamp et al. 1984). In own experiments analyzing isolated *Arabidopsis* mitochondria the K_i for the inhibition of the respiratory chain was determined to 1.3 µM H_2S (data not shown). The low K_i value demonstrates the sensitivity of the cell for toxic effects caused by H_2S. The cellular concentration of H_2S in the fumigated plants was not determined. To investigate the involvement of H_2S in SIR it would be very important to determine the internal H_2S concentration. Other methods to measure internal H_2S have to be developed, such as the use of amperometric H_2S microelectrodes. Toxic effects of elevated external H_2S concentrations might not reflect effects of increased local H_2S concentration, which might inhibit growth of pathogenic micro-organisms. In addition to the effect of H_2S causing oxidative stress one has to keep in mind that the plants were transferred from a 16 h light/8 h dark cycle at 60 µmol* m^{-2} *s^{-1} into continuous light of 250 µmol* m^{-2} *s^{-1}. Mild light stress can not be excluded.

One aim of this study was to investigate whether the OAS-TL isoforms localized in different compartments of the cell were differentially expressed when the plants were fumigated with

H_2S. One could assume that the mitochondrial isoform was induced because even low H_2S concentrations act toxic on the respiratory rate. However, the three genes investigated were not differentially regulated. Probably the expression pattern of all seven OAS-TL genes in *Arabidopsis* have to be analysed.

Effects of H_2S fumigation on non-sulfur metabolites

In spite of the relatively large standard deviations, it is obvious that there is a decrease in the OAS/NAS contents with increasing H_2S concentration and duration of treatment. In the H_2S fumigated plants the OAS/NAS contents are always lower than in the control plants. That seems to be reasonable as substrate depletion and corresponds to the higher contents of cysteine and glutathione observed. At higher concentrations of H_2S treatment, some OAS/NAS accumulation was demonstrated at the beginning of the fumigation. However, after 6 h of fumigation the OAS/NAS contents were already almost used up for the thiol synthesis. Comparable results were also obtained in sulfate-sufficient *B. oleracea* plants in an H_2S exposure experiment of up to 10 d (Buchner et al. 2004a). Interestingly, the OAS content in the shoot of sulfate-deprived, H_2S exposed *B. oleracea* increased substantially from day 6 onwards, though to a lower extent than in sulfate-deprived plants (Buchner et al. 2004a). Our results clearly indicate that the OAS/NAS contents are limiting for the synthesis of even higher levels of thiols in H_2S fumigated plants. In non-fumigated *Arabidopsis* plants not only OAS/NAS contents but also the internal concentrations of H_2S might be limiting for the biosynthesis of increased thiol levels. For further approaches aiming to increase thiol levels in plants, one should keep the availability of H_2S in mind. It could also serve as a signal molecule to control thiol levels.

Chapter 4

H_2S concentrations in plants detected by an H_2S microsensor

Introduction

H_2S emission by higher plants was first observed by DeCormis (1968). The H_2S release was measurable by cysteine degradation in spinach leaves and the green alga *Chlorella fusca* (Schmidt 1982; Schmidt & Erdle 1983). Pyridoxal-5'-phosphate (PLP)-dependent cysteine desulfhydrases (CDes) required for iron-sulfur (Fe-S) cluster biosynthesis catalyse the degradation of cysteine to pyruvate, ammonium and H_2S or to alanine and H_2S (Rennenberg et al. 1987; Zheng et al. 1993). The CDes enzyme activity was first described for the NifS protein from *Azotobacter vinelandii* (Zheng et al. 1993). Recently, NifS-like proteins were also identified in *Arabidopsis thaliana* localized in the mitochondrion (AtNFS1) and in the chloroplast (AtNFS2), respectively (Kushnir et al. 2001; Leon et al. 2002; Pilon-Smits et al. 2002). Specific L- and D-cysteine desulfhydrase activities could be separated by using DEAE-cellulose chromatography techniques (Schmidt & Erdle 1983). Rennenberg (1989) summarized possible pathways of H_2S synthesis emitted by higher plants in response to sulfate, sulfur dioxide, L-cysteine and D-cysteine.

Burandt et al. (2001) observed that the specific L-cysteine desulfhydrase enzyme activity increased with increasing plant age. It is also possible to conclude that the higher the sulfur content in the plants is, the lower the activity of H_2S-releasing enzymes, such as L-cysteine desulfhydrase (L-CDes). Different field surveys have shown that a good sulfur supply can increase the resistance of agricultural crops against fungal pathogens (Schnug et al. 1995a). The mechanisms of sulfur-induced resistance (SIR) are, however, not yet known. Volatile sulfur compounds are thought to play an important role in these mechanisms. H_2S might act as a fungicide therein (Beauchamp et al. 1984; Sekiya et al. 1982b). At the moment it is not known whether the H_2S emission takes place before or after cysteine biosynthesis or whether the H_2S emission is genetically controlled or directly correlated with the sulfur supply.

Experiments with detached leaves and leaf discs incubated with L-cysteine have shown that under these conditions H_2S is emitted (Sekiya et al. 1982a). Rennenberg et al. (1990) used a large 2,6-l volume gas exchange cuvette to measure volatile sulfur compounds via gas chromatography (GC) connected with a flame photometric detector. At least the light-

dependent H_2S emission could be calculated to approximately 1 nmol of H_2S per 1 mol H_2O transpiration for spruce trees (*Picea abies*). H_2S was emitted in response to sulfur dioxide or sulfite in a light-dependent process at rates varying between 0,8 and 8 nmol*h^{-1}*cm^{-2} leaf area (summerized in Rennenberg 1989).

A sulfide hydrogen microsensor might be used to measure H_2S concentrations in plant leaves or emitted by plants. Generally, this equipment is used for the detection of H_2S in bacteria and liquids (Schulz & Jørgensen 2001; Schulz & de Beer 2002). After having done H_2S fumigation experiments on *Arabidopsis* where the enzyme activity and gene expression was elevated (Riemenschneider et al. 2005b) it is challenging to learn more about H_2S concentrations in plants and H_2S emission under natural conditions. With the H_2S microsensor it should be possible to detect the H_2S concentration in plant leaves. This hopefully will help to evaluate the impact and the role of H_2S (release) in relation to SIR.

Material

Plant material

Arabidopsis thaliana (L.) Heynh., ecotype C24, plants were grown in the greenhouse on soil in a 16-h light/8-h dark rhythm at a temperature of 23/21°C. Plants of different ages, two to six weeks old, have been used for the measurements with a H_2S microsensor. Tobacco (*Nicotiana tabacum* L. cv. W5) plants grown under same conditions as *Arabidopsis* have been taken as comparison.

H_2S measurements with the microsensor

For the measurements a H_2S microsensor from Unisense (Aarhus, Denmark), tip size 10 µm, has been used. This sensor is connected to a high sensitivity picoammeter (Unisense PA2000) and the anode is polarised against the internal reference. H_2S from the environment is penetrating through the sensor tip membrane. The signal is generated and the picoammeter converts the resulting reduction current to a voltage signal. The microsensor is moved by a micromanipulator.

1 M KH_2PO_4 buffer, pH 7,5 without oxygen, has been used to stabilise the microsensor. For the standard curve different amounts of a 100 mM Na_2S solution in a range of 0 to 300 µM were added (for more information and the quantification with respect to temperature, pH and pA see http://www.unisense.com).

Because of the light sensitivity of the microsensor tip a cooled-light lamp was necessary for the plant measurements. For focusing on the leaf of interest a binocular has been used.

Arabidopsis leaves dissected with 0,5 to 2% agarose gel pieces, pH 7, were measured from the leaf upper side to the leaf under side. Measurements in the opposite direction also have been done. Different points on the leaves (see Fig. 1) and leaves of different ages (leaf 3 to leaf 9) have been chosen. Tobacco leaves (leaves 1 and 2) have been measured once in the same way.

Results

First, the standard conditions optimised for bacteria and liquids (Schulz & Jørgensen 2001; Schulz & de Beer 2002) had to be adapted to plant material. Therefore different conditions were tested before the variant with a gel piece under the leaves has been chosen (Fig. 1a). This gel piece should ensure that the thin microsensor tip will not be destroyed during the measurements. Dependent on the leaf age an *Arabidopsis* leaf could be up to 200 to 400 µm thick. The different cell layers are shown in Fig. 2. The upper epidermis with the cuticle is followed by a compact cell layer called palisade parenchyma. The third cell layer is the spongy layer where big intercellular spaces could collect different gases. The last cell layer is the lower epidermis with stomates. In this experiment the microsensor was put on the leaf surface as start point zero. The measurements were followed by 25 to 50 µm steps up and down through the tissue waiting after each step a few seconds. Measurements have been done started at the upper and the lower epidermis placed the microsensor on the leaf surface by hand looking through a binocular. One problem for the measurements is the cuticle. The microsensor can glue there. But also the preparation of the leaf on the gel piece without damaging the leaf and fix it there is difficult. Different agarose consistencies have been tested and resulted therein that gel pieces with higher agarose concentrations are necessary for fixing the position of the leaf. Gel pieces with lower agarose concentrations are helpful for fixing the leaf on the agarose boulder.

The first profile is from measurements in *Arabidopsis* (Fig. 3a). Measurements have been started at the leaf surface at the upper epidermis in depth 0 µm. 50 µm steps followed down up to 400 µm. In the first 100 to 150 µm 11 to 14 µM H_2S have been detected. In lower depth the H_2S concentration decreases consistently up to 1 µM H_2S. In the tobacco plant profile (Fig. 3b) the measurement took place in the same way. One of the profiles shows higher H_2S concentration, all the others show values comparable with the *Arabidopsis* profiles. For conclusion in *Arabidopsis* a H_2S concentration in a range of 4 to 10 µM H_2S was detected, in tobacco plants the general H_2S concentration was found to be in the same range.

a) b)

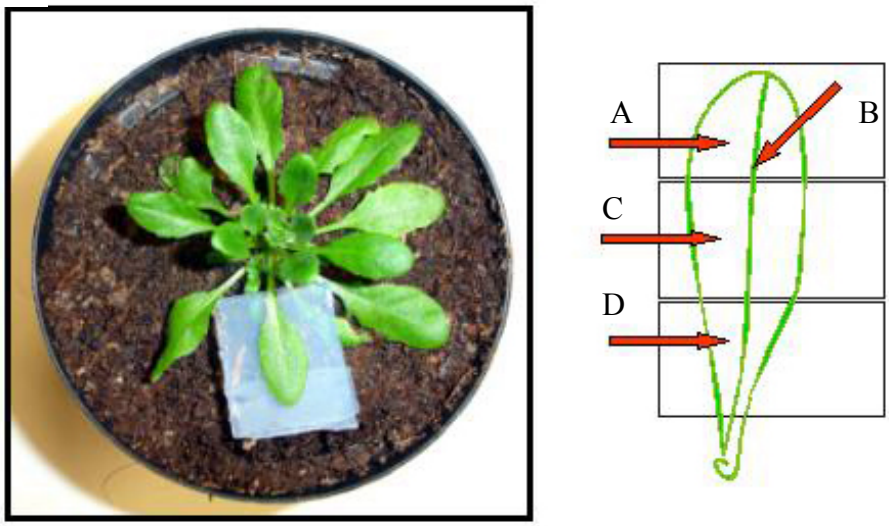


Fig 1: a) Four weeks old *Arabidopsis* plant with gel pieces under the leaves. The measurements took place through the leaf in the gel pieces to make sure not to damage the tip of the sensor. b) Graphic for showing different measurement points (A-D) on an *Arabidopsis* leaf. For the measurements the leaf is sectioned in three parts.

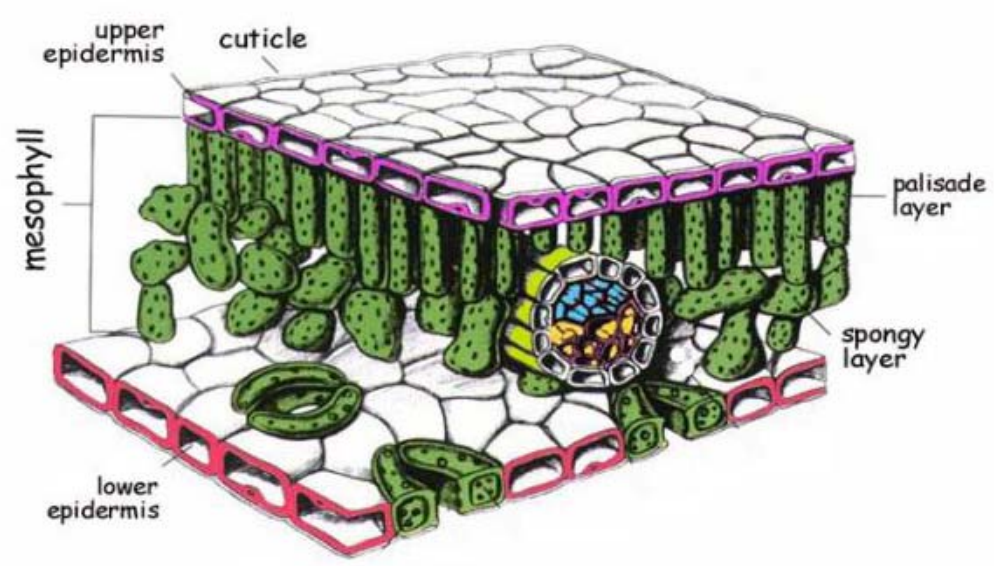
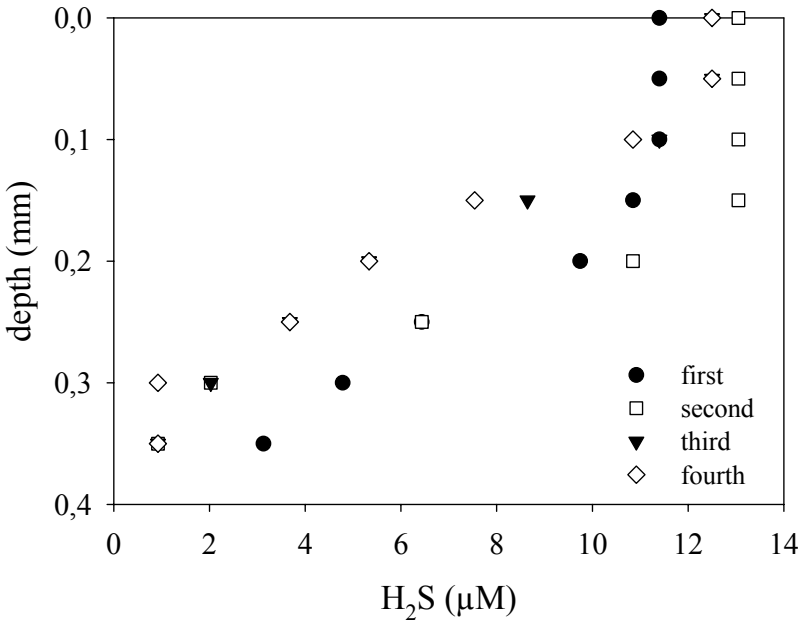


Fig 2: Scheme of a cross section through a leaf (www.borg.com/~lubehawk/photosyn.htm). The four different cell layers are shown. The mesophyll is surrounded by the upper and lower epidermis. Dependent on the leaf age an *Arabidopsis* leaf could be up to 200 to 400 µm thick.

a)



b)

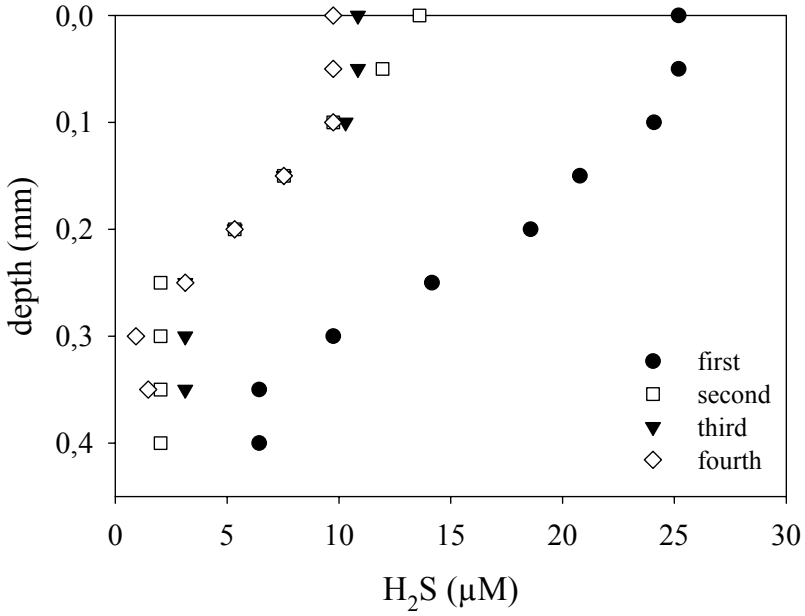


Fig 3: a) Results of one measurement at an *Arabidopsis* plant and b) at a tobacco plant. The detected H_2S concentration versus the depth is shown. The four different symbols show the results of four independent measurements in section A.

Discussion

Desulfhydrases are thought to be good candidates for H_2S release which might act against pathogen attack. To learn more about the physiological H_2S release, measurements with a microsensor normally used for H_2S detection in bacteria and liquid was used. The not fully established method shows one interesting possibility of measuring H_2S concentrations from plants under physiological conditions. The advantage of this method is the specific measurement points because of the small tip size. The tip size detects in seconds changes in the HS^- concentration. And the biggest advantage is the high sensitivity of this equipment to H_2S. The measured concentration in a range of 1 to 14 (tobacco 25) µM H_2S is in the same range as was calculated previously (pers. communication, Prof. Dr. A. Schmidt, Hanover).

This method made it possible to measure the H_2S content in plants punctured within a radius of 10 µm. Different points on one leave can be measured and also in different tissues. The equipment is quite sensitive against HS^- concentration and made it possible to detect lower H_2S concentrations then it would be possible with any other methods including analysis by GC. In all other methods used till date H_2S had to be collected in any way (Sekiya et al. 1982b; Rennenberg et al. 1990; McEwan & MacFarlane Smith 1998). Big volumes had to be collected to reach the H_2S concentration necessary for measurements at last with GC.

It is also necessary to keep in mind that there are some difficulties with the H_2S microsensor which make an exact calculation difficult. The first problem is the low reproducibility because of high deviations in independent measurements. It might be possible that hand-made microsensors cause this problem. Also the plants are quite individual; however, *in vivo* measurements are essential for further studies. Next, the tip of the microsensor is quite big and during the measurement it destroys whole cells. Typically a leaf of *Arabidopsis* is, dependent from the age, 200 to 400 µm thick. In the different cell layers the relations of apoplast to symplast are different. The pH differs in the different cell compartments (Burandt et al. 2002). During the measurements the different cell compartments are mixed and the pH is changed. The vacuole is normally the biggest cell compartment. The pH in the vacuole differs between pH 0,5 and pH 6 dependent on the compounds stored in it (e.g. Buchner et al. 2004b). Usually a pH profile has to be measured in the same place H_2S has been measured before.

The different layers atmosphere- leaf- gel constitute one more problem, because the system was originally developed for liquid environments. But there are new ideas how to measure more exactly. One possibility is to use a thinner microsensor tip with which it would be possible to focus more on the stomates and the respiration cave of the leaves. Then it might be

also possible to measure in one cell without damaging the membrane potential. H_2S concentrations in the cell and emission rates in the apoplast would be measurable. Rennenberg et al. (1990) was able to measure different H_2S emission rates via stomates. But for this idea a microscope and a really thin tip up to 0,2 µm had to be used. This implicates the next problem because such a tip size is currently not available in the Unisense system. Another possibility is to place a drop of buffer on the leaf for measuring the released H_2S solved in this drop. The problem here is the balance between the H_2S release of the leaf into the buffer and the atmosphere. Oil which can be used as a barrier can cause artifacts up to the damage of the microsensor. Keeping this in mind, measurements developed from standard methods have been started.

Taking all these results and ideas into account it is absolutely necessary to develop a system to measure H_2S release from plants *in vivo*. The development of a method combined with the already described method and other methods applied for ion quantification with microsensors could be helpful and should be one part planned for the future. Calculation and relevant measurements of the H_2S concentration necessary for stopping pathogens are also essential. Results from Riemenschneider et al. (2005b) have shown that H_2S concentrations in the atmosphere higher than 0,5 µl l^{-1} H_2S resulted in oxidative stress in *Arabidopsis* plants. So it is necessary to clarify if H_2S concentrations up to this concentration are high enough to stop or reduce pathogen attack. Bearing in mind all other possible compounds perhaps involved in pathogen protection (Salac 2005; Rausch & Wachter 2005), it is also important to think about an interaction of all these compounds acting together against pathogens.

Chapter 5

Sulfur supply and infection with *Pyrenopeziza brassicae* influence L-cysteine desulfhydrase activity in *Brassica napus* L.

Different field surveys have shown that sulfur (S) fertilization can increase the resistance of agricultural crops against fungal pathogens. The mechanisms of this sulfur-induced resistance (SIR) are, however, not yet known. Volatile S compounds are thought to play an important role because H_2S is toxic to fungi. A field experiment was conducted to analyse the influence of S fertilization and the activity of H_2S-releasing enzymes on fungal infections. Two levels of N and S fertilizers and two varieties of oilseed rape were investigated with respect to their potential to release H_2S by the enzymatic activity of L-cysteine desulfhydrase (LCD) and *O*-acetyl-L-serine(thiol)lyase (OAS-TL). LCD releases H_2S during cysteine degradation, while OAS-TL consumes H_2S during cysteine synthesis and free H_2S is only released in a side reaction. All plots of the field trial showed an infection with *Pyrenopeziza brassicae* and leaf disc samples were taken from visibly infected leaf areas and apparently uninfected areas to investigate the reaction to the infection in relation to the treatments. Different sulfur fractions and the activities of LCD and OAS-TL were measured to evaluate the potential to release H_2S in relation to sulfur nutrition and fungal infection. S fertilization significantly increased the contents of total S, sulfate, organic sulfur, cysteine, and glutathione in the plants, but decreased LCD activity. Infection with *P. brassicae* increased cysteine and glutathione contents, as well as the activity of LCD. Therefore crops were able to react to a fungal infection with a greater potential to release H_2S, which is reflected by an increasing LCD activity with fungal infection.

Introduction

The role of sulfur (S) in the resistance of crops against diseases became obvious at the end of the 1980s when atmospheric S depositions were so much reduced by clean air acts that sulfur deficiency became a widespread nutrient disorder in European agriculture (Booth et al. 1991; Kjellquist & Gruvaeus 1995; Knudsen & Pedersen 1993; Richards 1990) and the infection of crops with certain diseases became increasingly obvious (Paul 1992; Schnug & Ceynowa 1990; Schnug et al. 1995a). It has been long known that foliar applied elemental S has a

fungicidal impact but only recently could it be shown that soil-applied sulfur in the form of sulfate also had a significant effect on the health status of crops. A significant repressive effect of soil-applied S on the infection of oilseed rape with *Pyrenopeziza brassicae*, grapes with *Uncinula necator*, and potato tubers with *Rhizoctonia solani* was found (Bourbos et al. 2000; Klikocka et al. 2005; Schnug et al. 1995a). The results of these experiments indicate that S metabolites are involved in disease resistance and support the concept of sulfur-induced resistance (SIR) (Schnug et al. 1995a). The S metabolism of plants offers several possibilities to combat fungal attacks and different metabolites were investigated with respect to their role in SIR. For instance elemental S depositions in the vascular tissue of resistant cocoa (*Theobroma cacao*) in response to infection with *Verticillium dahliae* were attributed to the toxicity of elemental S (Cooper et al. 1996; Resende et al. 1996; Williams et al. 2002). Other mechanisms to combat biotic stress, which are provided by S metabolism, involve glutathione (GSH), phytoalexins, glucosinolates, and the release of S-containing volatiles (Haneklaus et al. 2004).

H_2S is cytotoxic and therefore a relationship between increasing H_2S emissions and the resistance of crops against pest and diseases is possible (Beauchamp et al. 1984; Schroeder 1993; Sekiya et al. 1982b). Atmospheric H_2S concentrations that are higher than 46 $\mu g\ m^{-3}$ (De Kok 1990) are reported to be phytotoxic, but there are no data available about fungitoxic H_2S concentrations. Haneklaus et al. (2006) calculated that a minimum uptake of 10 $\mu M\ H_2S\ h^{-1}$ by the pathogen would be necessary to yield a fungicidal effect.

The release of several volatile reduced-S compounds (hydrogen sulfide, carbonyl sulfide, dimethyl sulfide, carbon disulfide, and methylmercaptan) from various plant species have been identified (Schroeder 1993). Such measurements were mostly conducted with cut plant parts that were fed with concentrated sulfur solutions or with living plants under experimental conditions. No field data exist where the emission of gaseous reduced-S was measured from living plants under different nutritional conditions or in relation to a fungal infection. The mechanisms by which H_2S is released, the extent of the H_2S emission under natural conditions, and the relation to fungal diseases are not fully understood. Also the influence of the plant S supply on the amount of H_2S released by crops is not yet known, but in the case of other secondary S compounds, such as GSH and glucosinolates, significant positive relationships were found (Haneklaus et al. 1999; Schnug et al. 1995b). The emission of H_2S is a light-dependent process and can be induced by feeding plants with an excess of sulfur in the form of sulfur dioxide (Wilson et al. 1978), sulfate (Sekiya et al. 1982a; Winner et al. 1981)

or L-cysteine (Rennenberg 1989; Sekiya et al. 1982b). This indicates that the release of H_2S also depends on the sulfur nutritional status of the crop.

H_2S may be released prior to or after cysteine formation (Giovanelli 1990), but the question is still open as to which enzymes could be responsible for H_2S release. Two candidates are L-cysteine desulfhydrase (LCD) and *O*-acetyl-L-serine(thiol)lyase (OAS-TL). LCD catalyses the decomposition of cysteine to pyruvate, ammonia, and H_2S. OAS-TL is responsible for the incorporation of inorganic S into cysteine, which can be subsequently converted into other S containing compounds. H_2S is evolved in a side reaction (Tai and Cook 2000) and in a molar ratio the enzyme formed about 25 times more cysteine than H_2S mg^{-1} protein during the same incubation time (Burandt et al. 2001a). Therefore *in vitro* the reaction of OAS-TL is a net H_2S-consuming reaction. The studies of Burandt et al. (2001) gave the first indications of a relationship between the activity of potentially H_2S-releasing enzymes, the S status of the crop, and an infection with fungal diseases. Increasing total sulfur content in different genotypes of oilseed rape was associated with a decreasing LCD and an increasing OAS-TL activity and lower infection rates with *Verticillium dahliae*.

According to Giovanelli et al. (1980) and Schuetz et al. (1991) the OAS-TL activity was insensitive to changes in the sulfur supply and the LCD activity was not coupled to the emission of H_2S. Presumably, the evolution of H_2S in the presence of high concentrations of sulfate or sulphite results from a transitory state in which the incorporation of sulfide into cysteine does not keep pace with light coupled sulfate/sulphite reduction (Anderson 1990), as the addition of the physiological sulfide acceptor OAS inhibits the evolution of H_2S and enhances the production of cysteine (Filner et al. 1984; Rennenberg 1983b). On the other hand, compounds which inhibit the incorporation of cysteine into GSH, promote the evolution of H_2S, suggesting that when cysteine-consuming processes are inhibited and the concentration of cysteine increases, sulfide is emitted as H_2S (Rennenberg & Filner 1982). Therefore, it is still controversial if the enzymes LCD and OAS-TL, respectively, are responsible for the release of H_2S and if there is a relationship between the enzyme activities and the S nutritional status of the crops or fungal infections.

In this experiment the influence of S and N nutrition on different sulfur fractions and enzyme activities were investigated, because N and S show strong interactions in their nutritional effects on crop growth and quality due to their mutual occurrence in amino acids and proteins. The enzyme OAS-TL links the sulfur and the N assimilatory pathways as the precursor, OAS, is derived from the C and N assimilation pathways and the reaction product cysteine, may be

regarded as the primary organic compound containing reduced S (Warrilow & Hawkesford 1998).

It was the aim of this work to investigate the relationship between the S and N nutritional status of the crops, the activity of the enzymes LCD and OAS-TL, to analyse the relationship between a fungal infection, and to assess the activity of these enzymes in relation to the nutritional status of the crops.

Materials and methods

Experimental design of the field experiment

A multi-factorial field experiment was conducted in 2002 in Braunschweig (52°18' N, 10°27' E in Lower Saxony, Germany) on a loamy sand (dystric Cambisol/orthic Luvisol). The following factors were investigated in a completely randomized block design: (i) two varieties of winter oilseed rape (*Brassica napus* L.), one of which was susceptible to the fungus *P. brassicae* (Bristol) and a resistant variety (Lipton); (ii) two levels of sulfur (S) fertilization (0 and 150 kg ha^{-1} S); and (iii) two levels of nitrogen (N) fertilization (100 and 200 kg ha^{-1} N).

Each treatment had four replicates and the plot size was 60 m^2. For defining the growth stages (GS) of oilseed rape, the BBCH scale was used (Strauss et al. 1994). The S fertilization was applied as potassium sulfate (K_2SO_4) with potassium balance by KCl and split into three doses: two were applied in the autumn (40 kg S ha^{-1} before sowing at GS 01 and 40 kg S ha^{-1} at GS 12), and the third dose was applied in the spring at the start of vegetation together with the first N application (GS 19). N was applied as ammonium nitrate (NH_4NO_3) and the fertilization was split into two equal doses. The second N dose was applied at the start of main growth (GS 50–53). The experimental plots were infected with *P. brassicae* by evenly distributing infected straw material on the plots in the autumn (GS 11).

The leaf sampling was carried out at the start of main growth (GS 50–53) at the end of April 2002. Twenty to thirty fully developed young leaves from the upper third of the crops, and leaf discs from 15–20 fully developed young leaves of a diameter of 16 mm were taken and immediately frozen in liquid N and subsequently freeze-dried. All plots showed the early symptoms of infection with *P. brassicae* when the sampling was carried out: little white spots of conidia were visible on the leaves. Therefore, two kinds of leaf disc samples (15–20) from each plot were taken, one with visible symptoms of infection (white spots on the leaf material) and one without symptoms to investigate the influence of the fungal infection on sulfur metabolites and enzyme activities.

The infection with *P. brassicae* takes place in late autumn and the conidia are visible in early spring as little white spots on the leaf surface and bottom; later on they build necrotic spots of up to 2 cm diameter and the leaves show deformations. Typical symptoms are also necrotic areas at the stems and bursting of the stems.

Chemical analysis

Young, fully differentiated, leaves of oilseed rape were taken at the beginning of stem elongation. For mineral analysis, the samples were dried at 60°C in a ventilated oven until constant weight and finely ground (<0.12 mm) using a RETSCH ultra-centrifugal mill. For the determination of the total S content by X-ray fluorescence spectroscopy powdered material was prepared, mixing 1.1 g of plant material with 4.4 g of HOECHST wax C (Schnug & Haneklaus 1999). Total N was determined by employing the Kjeldahl method.

Sulfate was determined in freeze-dried plant material according to Novozamsky et al. (1986) and organic S was calculated as the difference between total sulfur and sulfate-S in the plant material.

In leaf disc samples, the GSH and cysteine content was determined as well as the activity of the LCD and OAS-TL. There was not enough leaf disc material to determine total N and S from this material, but differences in the mineral content were not likely.

Measurement of cysteine and glutathione

Twenty to thirty mg of fine-ground, freeze-dried plant material was diluted with 0.1 M HCl containing 4% polyvidone-25 (Hell & Bergmann 1990). The samples were centrifuged twice for 5 min each at 14.000 g at 4°C. Aliquots of the supernatants were neutralized with 0.08 M NaOH, reduced with 10 mM dithiothreitol, and the sulphydryl groups were derivatized with 10 mM bromobimane (Sigma-Taufkirchen, Germany) as described by Hell & Bergmann (1990). Separation, detection, and quantification of fluorescent adducts was achieved by a reversed phase column (Waters Nova-Pak C18, 4.6 x 250 mm) and a Hitachi HPLC System running with a gradient of 100% methanol and 0.1 M potassium acetate buffer as eluents.

Enzyme activity measurements

The activities of LCD and OAS-TL were determined in the frozen leaf disc samples as follows: the frozen plant material was ground in liquid N and the soluble proteins were extracted by adding 1 ml 20 mM TRIS/HCl, pH 8.0, to 100 mg plant material. After

centrifugation the protein content of the supernatant was determined according to Bradford (1976) using bovine serum albumin as a standard.

The OAS-TL assay contained a total volume of 1 ml: 5 mM OAS, 5 mM Na_2S, 3.33 mM dithiothreitol, 100 mM TRIS/HCl, pH 7.5, and 50 µl enzyme extract (Schmidt 1990). The solutions of OAS, Na_2S, and dithiothreitol were prepared at the start of the experiment. The reaction was initiated by the addition of Na_2S and the sample was incubated for 30 min at 37°C before the reaction was stopped by adding 1 ml acidic ninhydrin reagent (0.8% ninhydrin (w/v) in 1:4 concentrated HCl:HOAc) in order to determine the cysteine concentration (Gaitonde, 1967). The samples were heated at 100°C for 10 min and, finally, 2 ml EtOH was added to stabilize the colour complex. The absorption of the samples was measured at 560 nm in a micro plate reader (Fluostar Optima, BMG Labtechnologies, Offenburg). Solutions with different concentrations of L-cysteine were prepared, treated in the same way as the assay samples, and were used for the quantification of the enzymatically formed cysteine. The linearity of the product formation with respect to incubation time, and the amount of protein given to the single assay, was carefully tested.

The LCD activity was measured by the release of sulfide from cysteine in a total volume of 1 ml consisting of 2.5 mM dithiothreitol, 0.8 mM L-cysteine, 100 mM TRIS/HCl, pH 9.0, and enzyme extract. The reaction was initiated by the addition of L-cysteine. After incubation for 15 min at 37°C the reaction was terminated by adding 100 µl of 30 mM $FeCl_3$ dissolved in 1.2 N HCl and 100 µl 20 mM *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride dissolved in 7.2 N HCl (Siegel, 1965). The formation of methylene blue was determined in microtitre plates at 670 nm. Solutions with different concentrations of Na_2S were prepared, treated in the same way as the assay samples and were used for the quantification of enzymatically formed H_2S. For each treatment four samples were collected and in each sample the assays were repeated three times on separate days.

Statistical calculations

ANOVA was used to determine which means were significantly different from others at the 5% significance level employing the CoHort software package (Simons 1995). The analysis of variance was conducted with three factors (S and N fertilization, and cultivars). The influence of infection with *P. brassicae* on the investigated parameters was calculated by a one-factorial ANOVA.

Results

In Table 1 the influence of N and S fertilization, cultivars, and infection with *P. brassicae* on N and S contents, on different sulfur fractions, and enzyme activities is summarized. Both the S and N fertilization were effective as the mineral contents of both elements increased significantly with fertilization. This is a prerequisite to investigate nutritional effects on other components. The two cultivars *Bristol* and *Lipton* showed no differences with respect to the investigated components and they also showed no differences with respect to their susceptibility to the fungus *P. brassicae*.

Effect of S fertilization on different S fractions and the activity of LCD and OAS-TL

The S fertilization had a significant effect on all investigated S fractions. The total S content increased on an average by 4.5 mg g^{-1}. The S content in the control plots are an indication of latent S deficiency with a value of 4.8 mg g^{-1} S, therefore, the plants showed no symptoms of severe S deficiency, but the sulfur content was below the value for a maximum yield. The fertilized plots were sufficiently supplied with S (Schnug and Haneklaus 1998). The sulfate content in the leaves increased with S fertilization by 1.5 mg g^{-1} and the organic S fraction by 3.3 mg S g^{-1}. The GSH and cysteine content increased significantly, too, by S fertilization, but these fractions contributed little to the increase of the organic S (0.02 mg g^{-1} cysteine-S, 0.05 mg g^{-1} GSH-S), which mainly resulted from increasing protein contents. It could be demonstrated that, in plants which were fertilized with S, the LCD activity was significantly lower than without additional S fertilization. The OAS-TL on the other hand was not significantly influenced by S fertilization (Table 1) but also tended to a higher activity in unfertilized plots. Therefore, in plants which showed S deficiency, the activity of the cysteine synthesizing enzyme and the activity of the cysteine catabolizing enzyme was higher than in plants, which were supplied with sufficient S.

Table 1: Influence of N and S fertilization, and cultivars on L-cysteine desulphydrase and *O*-acetyl-L-serine(thiol)lyase activity and cysteine and glutathione content in leaf discs and total N and S, sulphate, and organic S contents in young, fully differentiated leaves of oilseed rape at the start of stem elongation (three-factorial ANOVA) and influence of infection with *P. brassicae* on these parameters (1 factorial ANOVA).
Tukey–Kramer test; different letters indicate statistically significant differences between means at the 5% probability level, standard deviation is given initalic letters.

Factor	N (mg g^{-1} DW)	S (mg g^{-1} DW)	Sulphate (mg g^{-1} DW)	Organic S (mg g^{-1} DW)	Cysteine (µmol g^{-1} DW)	Glutathione (µmol g^{-1} DW)	L-cysteine desulphydrase (nmol H_2S mg^{-1} protein min^{-1})	*O*-acetyl-L-serine(thiol)lyase (nmol cys mg^{-1} protein min^{-1})
S fertilization								
0	46.2 a	4.78 b	0.95 b	3.83 b	0.73 b	12.1 b	15.6 a	2422 a
s.d.	*5.0*	*0.46*	*0.19*	*0.45*	*0.36*	*3.6*	*4.3*	*1071*
150	45.2 b	9.29 a	2.48 a	6.80 a	1.20 a	13.6 a	13.4 b	2084 a
s.d.	*6.4*	*0.80*	*1.24*	*1.60*	*0.62*	*4.2*	*4.3*	*1030*
N fertilization								
100	40.53 b	7.00 a	1.96 a	5.03 b	0.95 a	12.1 b	12.7 b	1901 b
s.d.	*2.2*	*2.28*	*1.46*	*1.76*	*0.56*	*4.1*	*3.9*	*857*
200	50.65 a	7.07 a	1.47 b	5.60 a	0.98 a	13.7 a	16.3 a	2606 a
s.d.	*3.0*	*2.44*	*0.71*	*2.00*	*0.57*	*3.7*	*4.1*	*1130*
Cultivars								
Bristol	45.7a	6.93 a	1.71 a	5.22 a	0.99 a	12.3 a	14.0 a	2190 a
s.d.	*5.6*	*2.31*	*1.09*	*1.77*	*0.57*	*4.0*	*4.3*	*1071*
Lipton	45.7 a	7.14 a	1.73 a	5.41 a	0.94 a	13.5 a	15.0 a	2316 a
s.d.	*5.8*	*2.40*	*1.25*	*2.03*	*0.56*	*3.9*	*4.4*	*1053*
Infection with: *Pyrenopeziza brassicae*								
Yes	No data	No data	No data	No data	1.35 a	14.5 a	17.4 a	2279 a
s.d.					*0.45*	*3.7*	*3.7*	*1102*
No	No data	No data	No data	No data	0.58 b	11.2 b	11.6 b	2227 a
s.d.					*0.35*	*3.6*	*2.9*	*1024*

Effect of N fertilization on different S fractions and the activity of LCD and OAS-TL

Not only the S fertilization, but also the N fertilization, had a significant effect on the investigated S fractions; with the higher N dose the sulfate content in plant material decreased while the organic S pool increased, indicating that with higher N supply more proteins were metabolized and, therefore, more sulfate was converted into proteins. This process was also reflected by the enzyme activities: the OAS-TL activity significantly increased with N fertilization and, consequently, more cysteine was metabolized. On the other hand, the activity of the cysteine-catabolizing enzyme (LCD) also increased with N fertilization (Table 1).

Effect of infection with* P. brassicae *on different S fractions and the activity of LCD and OAS-TL

The influence of fungal infections on the investigated parameters is also summarized in Table 1: the cysteine content increased in the leaf disc samples 2.3-fold when a visible infection with *P. brassicae* was discovered, and GSH increased 1.3-fold. Both metabolites were metabolised to a higher extent in leaf areas, which were obviously damaged by the pathogen. Keeping in mind that OAS-TL is catalysing the cysteine synthesis, and cysteine being the substrate for degradation by LCD, fungal infection can be expected to influence both enzyme activities. While the LCD activity significantly increased with infection with *P. brassicae*, the OAS-TL activity increased only slightly and not significantly in the infected tissue. In Fig. 1 the LCD activity was plotted against the S content of the plants and the infected plants showed a distinctly higher enzyme activity with a mean value of 17.4, whereas uninfected plants had a mean activity of only 11.6 nmol H_2S mg^{-1} protein min^{-1}.

Correlations between the different S fractions and the enzyme activities

Correlations between different S compounds and enzyme activities are shown in Fig. 2. Weak, but significant relationships were found between the different S fractions investigated and the LCD activity. Close relationships were only found for the total S content and the organic S and sulfate, respectively, and between cysteine and GSH. There was a negative relationship between total S and sulfate-S with LCD while GSH and cysteine were positively correlated with the LCD activity, revealing that higher cysteine and GSH contents were related to a higher LCD activity. The N content in the plant material showed a highly significant positive correlation with the LCD activity and a weak correlation with OAS-TL. Both enzymes were positively related to each other with a correlation coefficient of r =0.47.

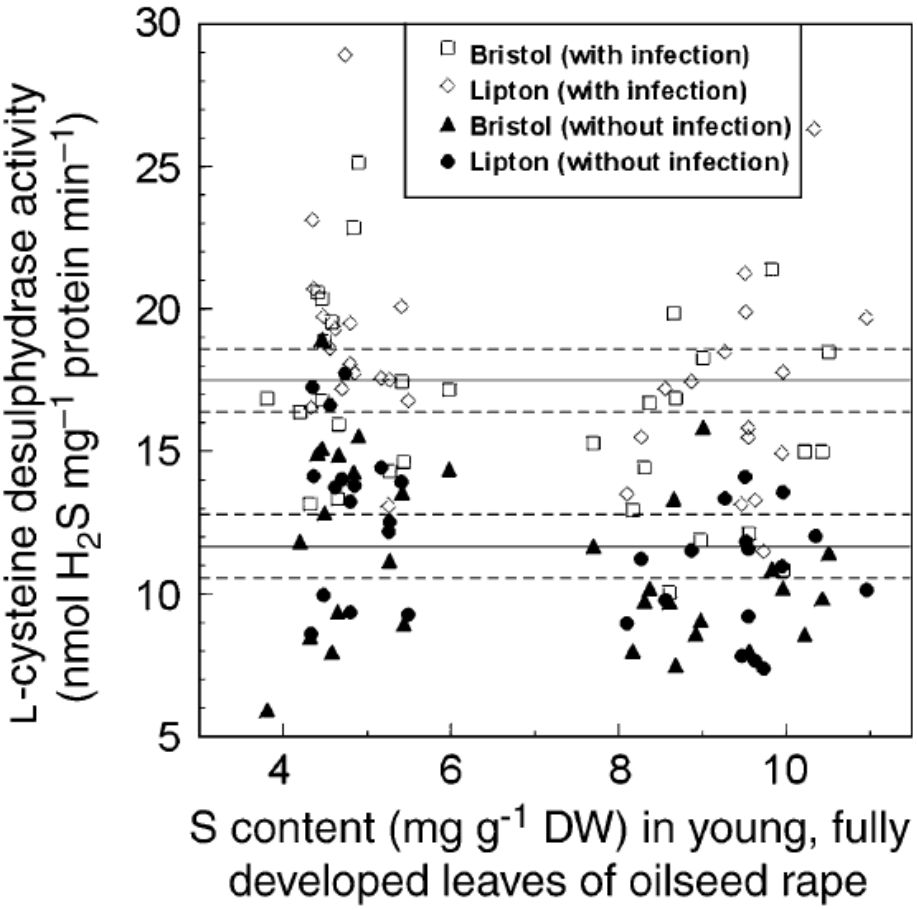


Fig. 1: Relationship between total sulfur content in young, fully differentiated leaves of oilseed rape at stem elongation and L-cysteine desulphydrase (LCD) activity in relation to infection with *P. brassicae*. [Note: continuous lines=mean values for LCD in infected (upper) and non-infected (lower) leaf discs (see Table 1); dashed lines=LSD5%].

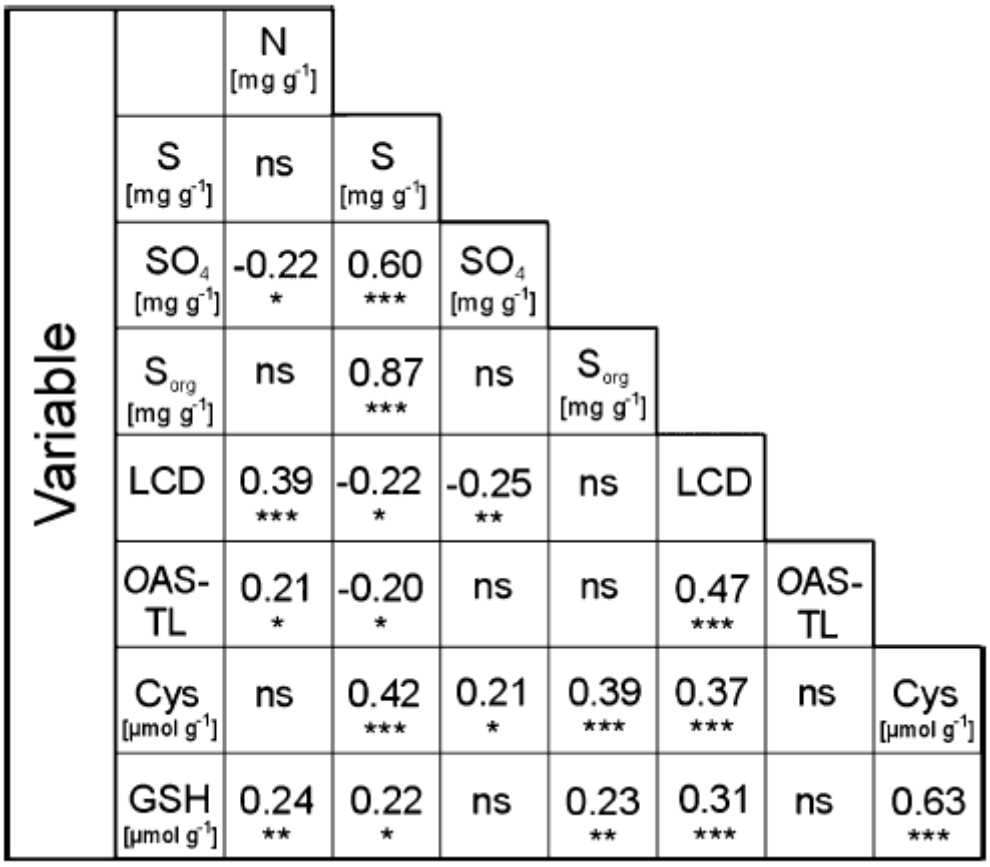


Fig. 2: Correlation coefficients (r) for the relationships between N and S contents, different sulfur fractions, and the enzyme activities in young, fully differentiated leaves and leaf discs of *Brassica napus* L. [LCD, L-cysteine desulphydrase activity (nmol H_2S mg^{-1}protein min^{-1}); OAS-TL, O-acetyl-L-serine(thiol)lyase activity (nmol cys mg^{-1}protein min^{-1}); Cys, cysteine, GSH, glutathione; ns, non-significant; and significant at *P<0.05, **P<0.01, and ***P<0.001].

Discussion

It was the aim of this work to investigate if there is a relationship between the S nutritional status of the crop, the potential of the plant to release H_2S, and fungal infections. The results clearly showed that correlations existed between all investigated S fractions and the S nutritional status. The correlations (Fig. 2) were mostly weak but highly significant. It is important to mention that these are results from a field experiment where there are always a great number of influencing factors. There are several diseases, insects, grazing animals like roe deer, and climatic conditions, which are influencing the crops. In pot experiments under controlled conditions such correlations are often much closer (De Kok et al. 1981; Schnug et al. 1995b). For a field experiment, weak but significant correlations are of high relevance to describe and understand the relationships between S fractions and metabolic processes.

With increasing S supply more free sulfate was available that could be incorporated into organic S, such as cysteine and GSH. Despite the very low proportion of cysteine and GSH

from the total organic S pool, both compounds seemed to be very important in the process of SIR because the contents of cysteine and GSH significantly increased when the plant material was visibly infected with *P. brassicae*. An increase in the pool of GSH has been measured in response to very different environmental stress factors like chilling, heat shock, pathogen attack, active oxygen species accumulation, air pollution, or drought (Dhindsa 1991; Kocsy et al. 1996; May et al. 1996, 1998; Nieto-Sotelo and Ho 1986; Sen Gupta et al. 1991).

Cysteine is the first stable organic S compound which is formed in the metabolism of S, and it is the precursor for all other S containing metabolites in the plant such as methionine, glucosinolates, and GSH (Warrilow & Hawkesford 1998). GSH has an important role in acting as a mobile pool of reduced S in the regulation of plant growth and development, and as an antioxidant in stress responses (Lamoureux & Rusness 1993; May et al. 1998; Noctor et al. 1998). Therefore, both are probably rapidly changing pools and act as the source for the metabolism of other S-containing compounds, which are important in SIR. Cysteine is the substrate of the LCD and, therefore, a rising S supply increased the substrate availability for the enzyme. However, while the results clearly showed an increase in LCD activity with fungal infection, S fertilization led to a decrease in the activity of LCD.

The OAS-TL activity was neither influenced by S nutrition, nor by fungal infection. Therefore OAS-TL is more likely to be regulated by the N assimilatory pathway because the OAS-TL activity significantly increased with N fertilization (Table 1) and the N content of the plant material (Fig. 2), and, consequently, more cysteine was metabolized. On the other hand, the activity of the cysteine-decomposing enzyme (LCD) also increased with N fertilization (Table 1), probably to prevent the plant from a too high and toxic cysteine pool. This is also reflected by a highly significant correlation between the N content of the plant material and LCD activity (Fig. 2). Several studies established regulatory interactions between assimilatory sulfate and nitrate reduction in plants (Brunold 1993; Koprivova et al. 2000; Yamaguchi et al. 1999). The two assimilatory pathways are interrelated; deficiency of one nutrient represses the other pathway. OAS, the precursor of cysteine, plays an important role in the regulation of sulfate uptake and assimilation. OAS seems to be limiting for cysteine synthesis in the presence of excess sulfate (Rennenberg 1983b). By comparison, OAS accumulates during S starvation and may thus act as a signal of the S status (Kim et al. 1999). OAS acts most probably as a transcriptional regulator, since higher OAS contents strongly increased mRNA levels of adenosine-5'-phosphosulfate reductase (APR), sulfite reductase, chloroplastic OAS-TL, and cytosolic serine acetyltransferase (Kopriva & Koprivova 2003).

Both enzymes, OAS-TL and LCD showed a positive correlation (Fig. 2) in infected as well as uninfected leaf discs. Burandt et al. (2001) found an inverse relationship of both enzyme activities for different genotypes of oilseed rape. These crops received the same rate of S fertilization but differed in their susceptibility to different fungal diseases. This stresses the significance of genetic differences and putatively involved modifications of S metabolism.

The fact that the activity of OAS-TL and LCD were higher in S-deficient plants indicates that, under conditions of S deficiency, S metabolism is activated and the participating enzymes are up-regulated. There are two possible explanations: the deficient plants were more susceptible to fungal diseases and, therefore, they increased metabolic pathways which were involved in plant protection. The second explanation could be that the S-deficient plants already had a stronger fungal infestation and the mechanisms of S-induced resistance were activated. Therefore the release of H_2S can be a mechanism of protection to prevent a fungal attack, or the answer to a fungal attack, or perhaps both mechanisms work at the same time.

The data in Fig. 1 clearly reveal that the activity of LCD was more strongly influenced by the infection status of the crops than by S nutrition. The LCD activity increased by about 50% due to infection with *P. brassicae*. The fact that OAS-TL was not significantly up-regulated while the product of the reaction, cysteine, increased strongly, probably shows that the enzyme activity was high enough to allow a fast turnover from sulfate to cysteine. These results suggest that OAS-TL is not actively increasing the H_2S release with infection, but can only participate in an increasing H_2S release in a passive way. By contrast, LCD seemed to be an enzyme whose activity is directly induced by an infection with *P. brassicae*, and therefore a higher H_2S release due to a higher LCD activity is possible. This is a strong hint that the evolution of H_2S could be an important strategy of the plant to combat a fungal attack. The positive relationship between OAS-TL and LCD indicates that the activity of OAS-TL is also increasing after fungal infection, but not as a direct result of the infection, rather as a reaction to the activity of LCD which is consuming cysteine, the product of the OAS-TL reaction.

The finding that LCD activity was significantly increased in infected plant tissue is a strong indication that the release of H_2S, is correlated with the fungal infestation of the crop. Therefore, the mechanism of H_2S release as a mechanism to increase the natural resistance of the crops against fungal infestations seems to be an important part of the SIR. In addition, the cysteine content increased by more than 100% and GSH by about 30% in infected plant tissue. The influence of the fungal infection on the cysteine and GSH content and LCD activity was even stronger than that of the S nutritional status of the crop, which also had a significant positive effect on the different S fractions. These results support the concept of

SIR. With a better S supply more cysteine and GSH are metabolized and, therefore, the potential of SIR is increasing.

In a further step, it will be necessary to measure the evolution of H_2S from living plants in relation to the enzyme activities in order to discover if the H_2S evolution of the crop is a strategy to combat a fungal attack**.**

Chapter 6

Expression of desulfhydrases and determination of their enzyme activity in *Brassica napus*

Introduction

Oilseed rape or canola (*Brassica napus* L.) belongs to the Brassicaceae family. In the "Triangle of U" (cited in Maluszynska & Hasterok 2005) the relationship between the different members of this family is described. *B*. *napus* is an amphidiploid bastard of *Brassica rapa* and *Brassica oleracea*. The genome of *B. napus* contains 19 chromosomes and is a mixture of the initial plants (n = 10 + 9). Because of using *B. napus* for animal feed, vegetable oil and also as a renewable resource, for example biodiesel, the handling with oilseed rape became more and more interesting.

Enhanced emission controls led to a dramatic reduction in atmospheric deposition of sulfur in recent years (Dämmgen et al. 1998). This change has had an important impact on agriculture and in the 1980's sulfur deficiency symptoms have been observed especially at oilseed rape (Schnug et al. 1995a). This and observations of an increase of diseases led to the opinion that there might be a relationship between the sulfur supply, the high sulfur demand of rape (Holmes 1980) and defence mechanisms against diseases like fungi attack. The hypothesis of sulfur-induced resistance (SIR) was invented.

The physiological background for the natural resistance of crops against certain pests and diseases might be related to increasing H_2S emission of the plants, which is highly toxic and thus may act as a fungicide (Beauchamp et al. 1984; Sekiya et al. 1982a).

H_2S may be released before or after cysteine formation (Giovanelli 1990). Two candidates which could be responsible for H_2S release are cysteine desulfhydrases (CDes) and *O*-acetyl-L-serine(thiol)lyases (OAS-TL). CDes catalyses the degradation of cysteine to pyruvate, ammonium and H_2S or to alanine and H_2S (Rennenberg et al. 1987; Zheng et al. 1993). OAS-TL is responsible for the incorporation of inorganic sulfur into cysteine, which can be subsequently converted into other sulfur-containing compounds. H_2S could be evolved in a side reaction (Burandt et al. 2001c). But also glucosinolates, phytoalexines, elemental sulfur and glutathione (GSH) might be involved in plant protection against pathogens (Rausch & Wachter 2005).

Results from Burandt et al. (2001a) indicate that the capability to use available soil sulfur is genetically controlled. The higher the sulfur content in plants, the lower the L-cysteine desulfhydrase activity and the higher the OAS-TL activity. Infections with *Verticillium dahliae* have shown a relationship between the sulfur nutrition of *B. napus* and *V. dahliae*, with higher total sulfur content the plants show less infestation with the fungus.

Field experiments with *B. napus* and *Pyrenopeziza brassicae* have shown an increase of the thiol concentration and the L-cysteine desulfhydrase activity. Therefore crops were able to react to a fungal infection with a greater potential to release H_2S, which is reflected by an increasing L-cysteine desulfhydrase enzyme activity with higher fungal infection (Bloem et al. 2004).

Because of the commercial interests and the observations and results from the last years we would like to focus more on the H_2S releasing enzymes in *B. napus*. Therefore some expression analyses of CDes under sulfur starvation and pathogen attack and enzyme measurements including the analysis of *B. napus* CDes sequences have been done.

Methods

Growth and harvest of plants

Brassica napus (line Lion and genotype 15157) seeds were germinated on towel paper in a climate-controlled room at 23°C. Three-day-old seedlings were transferred into cut Eppendorf tubes and placed in a hydroponic system. 25% Hoagland medium has been used for 18 days then half of the plants were transferred to Hoagland medium without sulfur. After three days leaves, stems and roots were harvested for the Northern blot analysis.

25% Hoagland medium was prepared with 1,25 mM $Ca(NO_3)_2$, 1,25 mM KNO_3, 0,5 mM NH_4NO_3, 1 mM $MgSO_4$, 0,25 mM KH_2PO_4 and 22,5 µM FeEDTA as final concentration supplemented by 1 ml microelements. The microelements' solution contained 80 µM $CuCl_2$, 11,6 mM H_3BO_3, 2,29 mM $MnCl_2$, 130 µM Na_2MoO_4 and 200 µM $ZnCl_2$. For sulfur starvation $MgSO_4$ was replaced by $MgCl_2$.

B. napus seeds from winter rape (line 1, 4, 6 and 7; Burandt et al. 2001a) have been germinated and then grown for 6 weeks in soil, infected with *Pseudomonas syringae* pv. *maculicola*, *P. syringae*. pv. *tabacci* and *E. carotovora* sp. *carotovora* in a concentration of 10^8 bacteria*ml^{-1}. The first two pathogens have been infiltrated with a syringe in leaves, control plants have been filtrated with $MgCl_2$. Plants infected with *E. carotovora* sp. *carotovora* have been damaged carefully with a needle and *E. carotovora* suspension has

been dropped on the damaged place. Control plants have been treated with $MgCl_2$. After ten days half of the infiltrated leaves have been harvested and stored in –70°C.

B. napus leaves from 24 days old plants of winter rape lines 1, 4, 6 and 7 (Burandt et al. 2001a) have been used in humid-chamber-tests with *Verticillium dahliae*. Three or four leaves per line have been covered with mycelium pieces (cylinders of 5 mm diameter and 4 mm height). The mycelium pieces were watered with 25 µl sterile H_2O. Influences from the fungus on the leaves have been observed over 16 days at RT under natural day-/night light conditions.

Cloning procedures

RNA was extracted from oilseed rape line 1 and 4 and transcribed in cDNA via RT-PCR using a kit from MBI Fermentas (St. Leon-Roth, Germany). For the amplification of *BnNifS* primer 354 5'-GGATCCACGTCTAAGGCTGTCTTC-3' extended by *Bam*HI as restriction site and primer 355 5'-CTGCAGGTGCTGAGACCACTGTAT-3' extended by *Pst*I as restriction site, for *BnL-CDes*I primer 358 5'-GCGGCGGATCGGAACGGC-3' and primer 359 5'-CTCATCGTTCGAACTCACAGG-3' have been used. For *BnD-CDes* primer 363 5'-AGATCTAGAAGACGGCTCTTAACACTC-3'/ *Bgl*II and primer 364 5'-GGTACCAATTGGACCACCACTTGCACA-3'/ *Kpn*I have been used. For the amplification of *BnL-CDesII* primer 357 5'-CGGATGAGAAGATGAAAAAGG-3' and a 3'- RACE primer have been used. The PCR tubes contained 0,2 mM dNTPs (Roth, Karlsruhe, Germany), 0,4 µM of each primer (MWG, Ebersberg, Germany), 0,75 µl RedTaq DNA-Polymerase (Sigma, Taufkirchen, Germany), and 5 µl template DNA in a final volume of 50 µl. Before starting the first PCR cycle, the DNA was denatured for 180 s at 94°C followed by 35 PCR cycles conducted for 60 s at 94°C, 60 s at 56°C, 58°C, 54°C and 54°C, respectively, for the different sequences, and 60 s at 72°C. The process was finished with an elongation phase of 420 s at 72°C. The amplified PCR fragments were ligated into pGEM-T/ pGEM-T_{easy} (Promega, Mannheim, Germany) and were introduced into *E. coli* strain XL1-blue.

RNA extraction and Northern blot analysis

Total RNA was extracted essentially as described (Chomczynski & Sacchi 1987). RNA samples (between 15 and 25 µg dependent on the abundance of the respective mRNA) were separated on 1% denaturing agarose-formaldehyde gels. Equal loading was controlled by staining the gels with ethidium bromide. After RNA transfer onto nylon membranes, filters

were probed with digoxigenin labelled cDNA probes obtained by PCR. To amplify the respective probes the sequence specific primers described above were used.
Colorimetric or chemiluminescent detection methods with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) or with CDP Star (Roche, Mannheim, Germany) as substrates for alkaline phosphatase were applied.
Total RNA from roots, stems and leaves was also isolated by another method based on Verwoerd et al. (1989), which involved an additional phenol-chloroform-isoamyl alcohol extraction of the aqueous phase after the first centrifugation. Northern blot hybridization of the desulfhydrase transcripts was performed according to Church & Gilbert (1984) with pre-hybridization/hybridization at 65°C. 10 µg of total RNA per slot was separated on an agarose/formaldehyde gel and blotted onto positively charged nylon membrane. cDNA fragments were labelled with ^{32}P-dCTP and used as hybridization probes. After hybridization the membrane was washed under high-stringency conditions and exposed to Kodak BioMax MS film (Kodak, Hemel Hempstead, United Kingdom).

Determination of enzyme activity

Plant material was ground with a mortar and pestle in liquid nitrogen and the soluble proteins were extracted by adding 1 ml 20 mM Tris/HCl, pH 8.0, to 100 mg plant material.
L-Cysteine desulfhydrase activity was measured by the release of H_2S from L-cysteine in the presence of DTT. The assay contained in a total volume of 1 ml: 0,8 mM L-cysteine, 1 mM DTT, 100 mM Tris/HCl, pH 9,0, and 100 µl protein solution. The reaction was started by the addition of L-cysteine; after incubation for 15 min at 37°C the reaction was terminated by adding 100 µl of 30 mM $FeCl_3$ dissolved in 1,2 N HCl and 100 µl 20 mM *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride dissolved in 7,2 N HCl (Siegel 1965). The formation of methylene blue was determined at 670 nm. Solutions with different concentrations of Na_2S were prepared, treated in the same way as the assay samples, and were used for the quantification of enzymatically formed H_2S. D-cysteine desulfhydrase activity was determined in the same way with following modifications: D-cysteine instead of L-cysteine was used; the pH of the Tris/HCl buffer was 8,0. The OAS-TL activities were determined as described using 50 µl protein extract (Burandt et al. 2001c). Protein contents were determined according to Bradford (1976) using BSA as protein standard.

Measurements of thiols and sulfate

For the quantification of cysteine and glutathione the HPLC method described in Riemenschneider et al. (2005b) has been used. For the analysis of the sulfate content in the plant material ion chromatography described in Blake-Kalff et al. (1998) has been done.

Sequence search

Sequences homologous to *Arabidopsis thaliana* desulfhydrase DNA sequences have been searched in the *B. oleracea* database (http://atidb.org). In these sequences degenerated primers have been designed and used for RT-PCR. The amplified sequences have been sequenced (MWG). Homologous protein sequences, belonging to the defined protein families pfam 00266 and pfam 00291, from *Arabidopsis*, rice (*Oryza sativa*) and the published *B. napus* sequences, translated in peptide sequences (http://www.ebi.ac.uk/emboss/transeq/), have been used to create a phylogenetic tree with CLUSTALW.

Results

Enzyme activity in infiltrated* Brassica napus *plants

To get an idea in which way different *B. napus* lines, characterized from Burandt et al. (2001a) react on pathogen attack, the plants were infiltrated with *P. syringae* pv. *tabaci*, *P. syringae.* pv. *maculicola* and *E. carotovora* sp. *carotovora* without determine the sulfur content again. All four lines show higher L-cysteine desulfhydrase and OAS-TL enzyme activity (Fig. 1). Slight differences were observed in the controls among each other. Therefore the larger differences between the reactions on the three bacteria are quite interesting. Line 1 with the highest sulfur content (Burandt et al. 2001a) has the lowest increase in H_2S release. In contrast to line 1, line 4 with the lowest sulfur content shows the highest capacity to emit H_2S. The release of H_2S is 142 nmol formed H_2S (mg protein x min)$^{-1}$, 3,5 times higher than in the corresponding control. Compared with all results infiltration experiments with *P. syringae* pv. *maculicola* show the highest reaction in the capacity of H_2S release in all lines. Interestingly, plants infiltrated with *P. syringae* pv. *tabaci* show the highest capacity to form cysteine. The whole OAS-TL enzyme activity is more inconsistent in all four lines. There are more variability observed in the different oilseed rape lines compared to the L-CDes enzyme activity.

Pathogen studies in humid chamber

One experiment to investigate the reaction of plant material on fungi was done in the humid chamber. In this case leaves of the four different oilseed rape lines and *V. dahliae* have been used. The experiment was observed over 16 days. Six different photographs taken after 5, 7, 9, 12, 14 and 16 days are shown from each *B. napus* line (Fig. 2). After five days line 7 shows most of chlorotic leaves and the production of anthocyanes is also visible in lines 1 and 7. Begin of necrosis could also be observed. After nine days the leaves of lines 1, 4 and 7 became more chlorotic. Following over the whole experimental period of 16 days lines 1 and 4 show the highest infection with *V. dahliae* on one leaf per chamber whereas on lines 6 and 7 no strong visible fungi growth was observed.

Sequences of desulfhydrases in oilseed rape

It was possible to construct primers to amplify parts of putative *B. napus* desulfhydrase sequences via RT-PCR. The length of the amplified *BnNFS1* DNA fragments is 1.364 bp, for *BnL-CDesI* 522 bp, for *BnNFS2* 416 bp and for *BnD-CDes* 753 bp. *Arabidopsis* sequences of all PLP-dependent desulfhydrases have been used to search for homologous sequences in rice (*O. sativa*). The respective accession numbers are given in Fig. 3. The phylogenetic tree shows all desulfhydrases of the three plant species to date. The *B. napus* protein sequences show between 80 to 88% identity and 87 to 90% similarity to *Arabidopsis* protein sequences. Only BnNFS2 shows higher identity and similarity to AtD-CDesI than to AtNFS2.

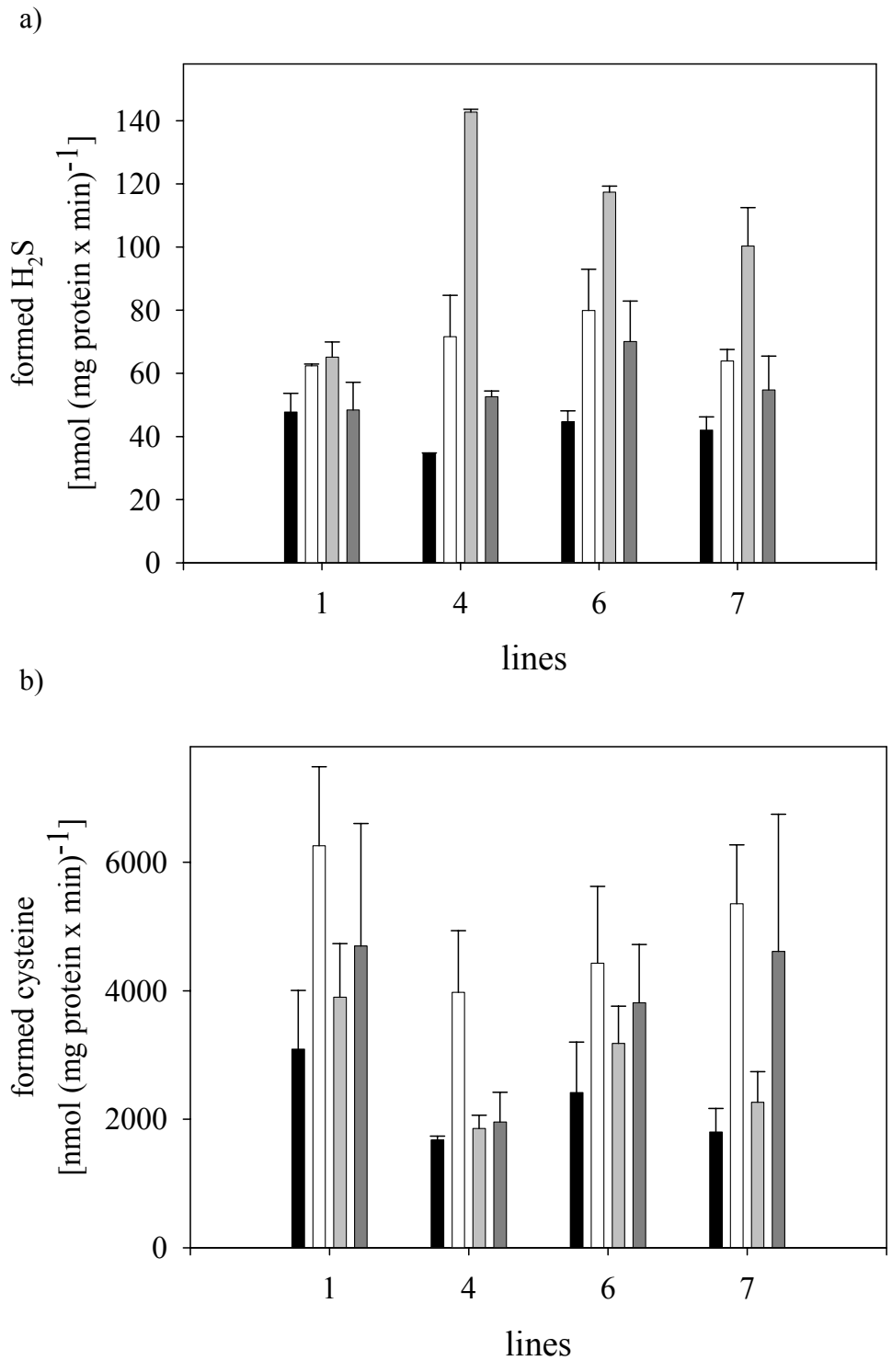


Fig 1: a) L-cysteine desulfhydrase enzyme activity, measured in infiltrated *Brassica napus* plants. The black bar shows the control, the white bar infection with *Pseudomonas syringae* pv. *tabaci,* light grey bar infection with *P. syringae* pv. *maculicola* and the dark grey bar infection with *Erwinia carotovora* sp. c*arotovora*; b) shows the OAS-TL enzyme activity measured in the same plant material described in a).

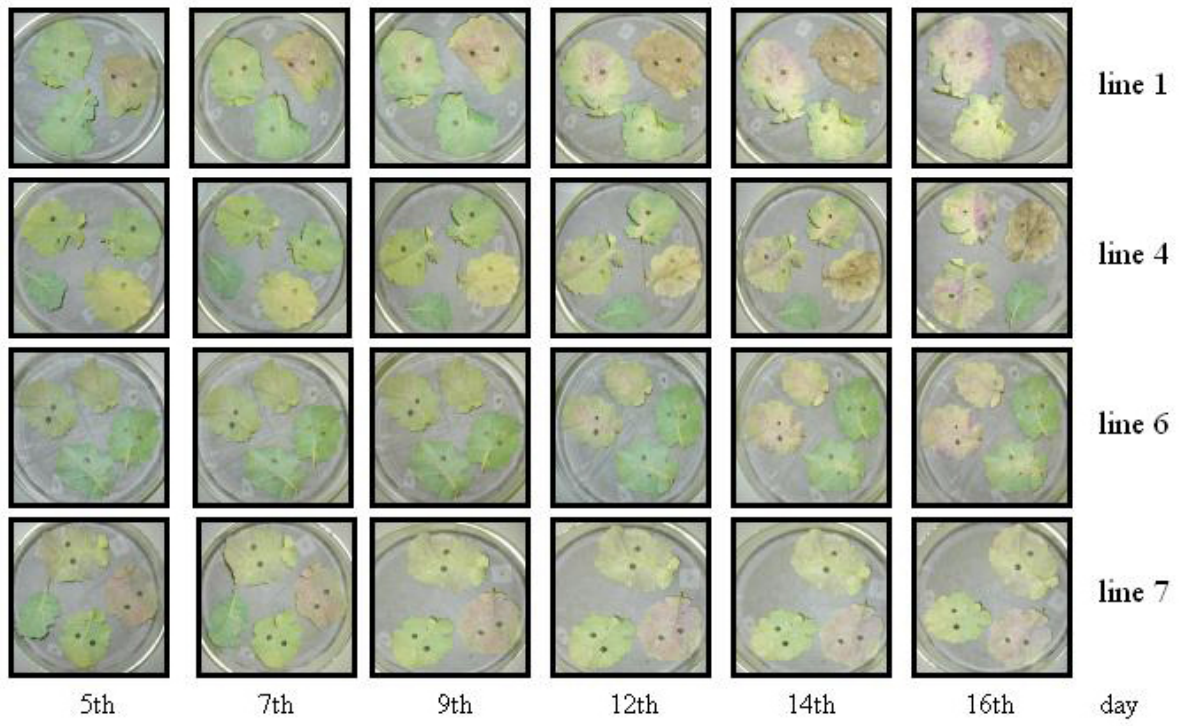


Fig 2: Plant leaves of different lines of oilseed rape in a humid chamber infected with *Verticillium dahliae* mycelium pieces. The experiment took place over 16 days under natural day-/night light conditions at RT.

Expression studies

For expression studies two different new *B. napus* lines have been chosen, Lion and genotype 15157, where the genotype 15157 is supposed to be more sensitive against pathogen attack (pers. communication, Silvia Haneklaus, Federal Agricultural Research Centre, Braunschweig, Germany). First it was interesting to know more about the general expression pattern of desulfhydrases under sulfur starvation in different organs. Therefore Northern blot analyses have been carried out (Fig. 4). Probes against L-CDesI, NFS2, D-CDes and NFS1 have been used. *L-CDesI* shows the highest mRNA accumulation in stems in line Lion and in genotype 15157 in plants grown under normal sulfur supply. The highest mRNA accumulation for *NFS2* can be observed for leaves. There seem to be more mRNA accumulation in leaves in line Lion under sulfur starvation. A higher accumulation of *D-CDes* mRNA is found in stems than in roots or leaves. In the case of *NFS1* the highest mRNA accumulation can be observed in line Lion in stems, followed by leaves and roots. In genotype 15157 the *NFS1* mRNA is highest accumulated in stems followed by roots and then leaves. The sulfate transporter *4.2*. (*ST 4.2.*) which has been used as an indicator for sulfur starvation (Buchner et al. 2004a) is indeed only expressed in plants grown in medium without sulfur. Interestingly, there is a different mRNA accumulation observed in line Lion and genotype

15157. Line Lion shows the highest mRNA accumulation in leaves under sulfur starvation. Genotype 15157 shows the highest mRNA levels in roots.

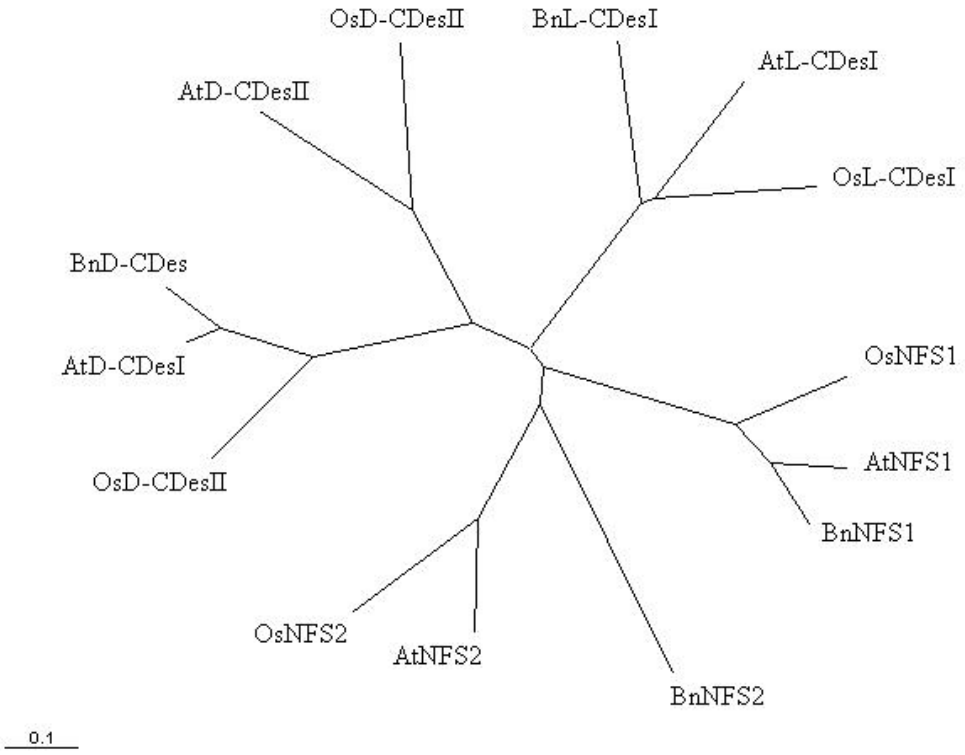


Fig 3: Phylogenetic tree of desulfhydrase protein sequences from *Arabidopsis thaliana*, *Oryza sativa* and *Brassica napus*. Sequences searched in BLAST using the *Arabidopsis* sequences as query were used to create a tree showing the different desulfhydrases in the three plants plants. The species and the respective accession numbers are given: *Arabidopsis thaliana*; AtNFS1O49543, AtD-CDes NP_175275, AtD-CDesII NP_189241, AtL-CDesI NP_191772, AtNFS2 AAM19798, *Oryza sativa*; OsNFS1 BAD28706, OsD-CDesI BAD16875, OsD-CDesII NP_917071, OsL-CDesI NP_908484, OsNFS2 ABA97759, *Brassica napus*; BnNFS2 136 aa, BnL-CDesI 174 aa, BnNFS1 453 aa, BnD-CDes 258 aa (this work).

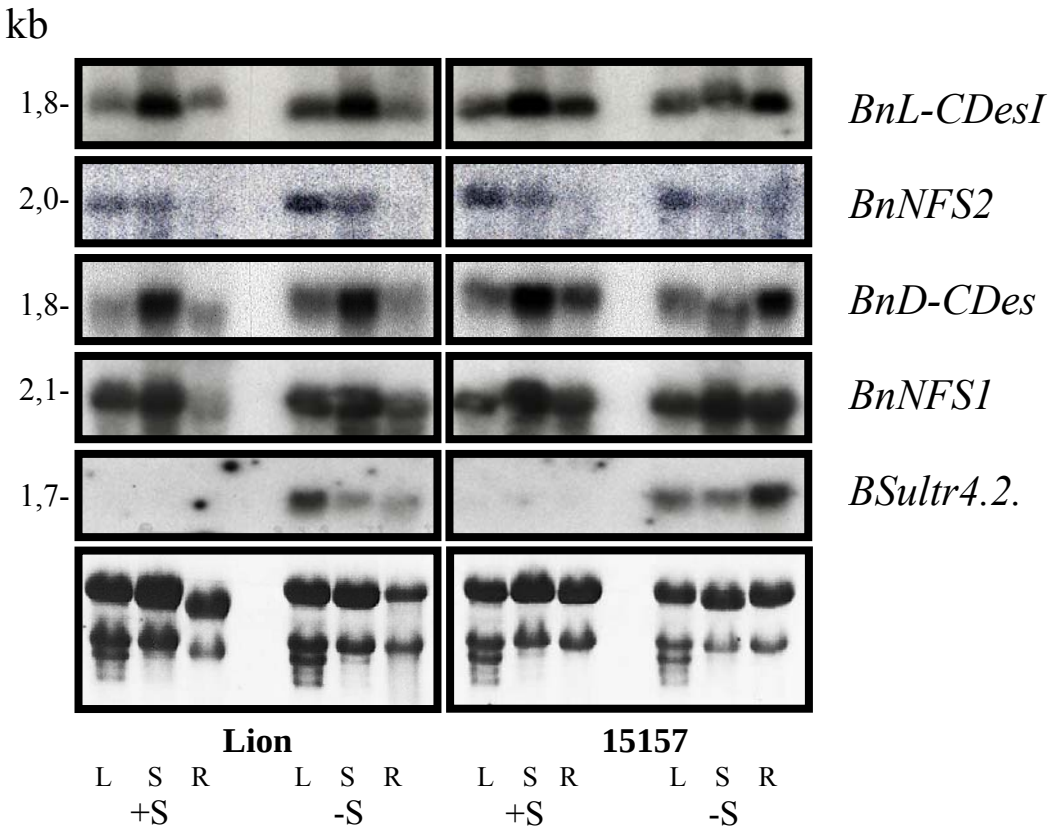


Fig 4: Northern blot analysis of the *Brassica napus* lines Lion and genotype 15157. Total RNA was extracted and 10 to 20 µg were loaded in each lane and blotted. To prove equal loading of the RNA the ethidium bromide stained gel is shown at the bottom. Probes were labelled with digoxiginin by PCR or with ^{32}P-dCTP. Probes from all four CDes and from *B. oleracea* sulfate transporter (*BSultr*) *4.2.* have been used. L, leaves; S, stem; R, roots. +S, plants grown in 25 % Hoagland medium with sulfur; -S, plants grown in medium without sulfur for 3 days.

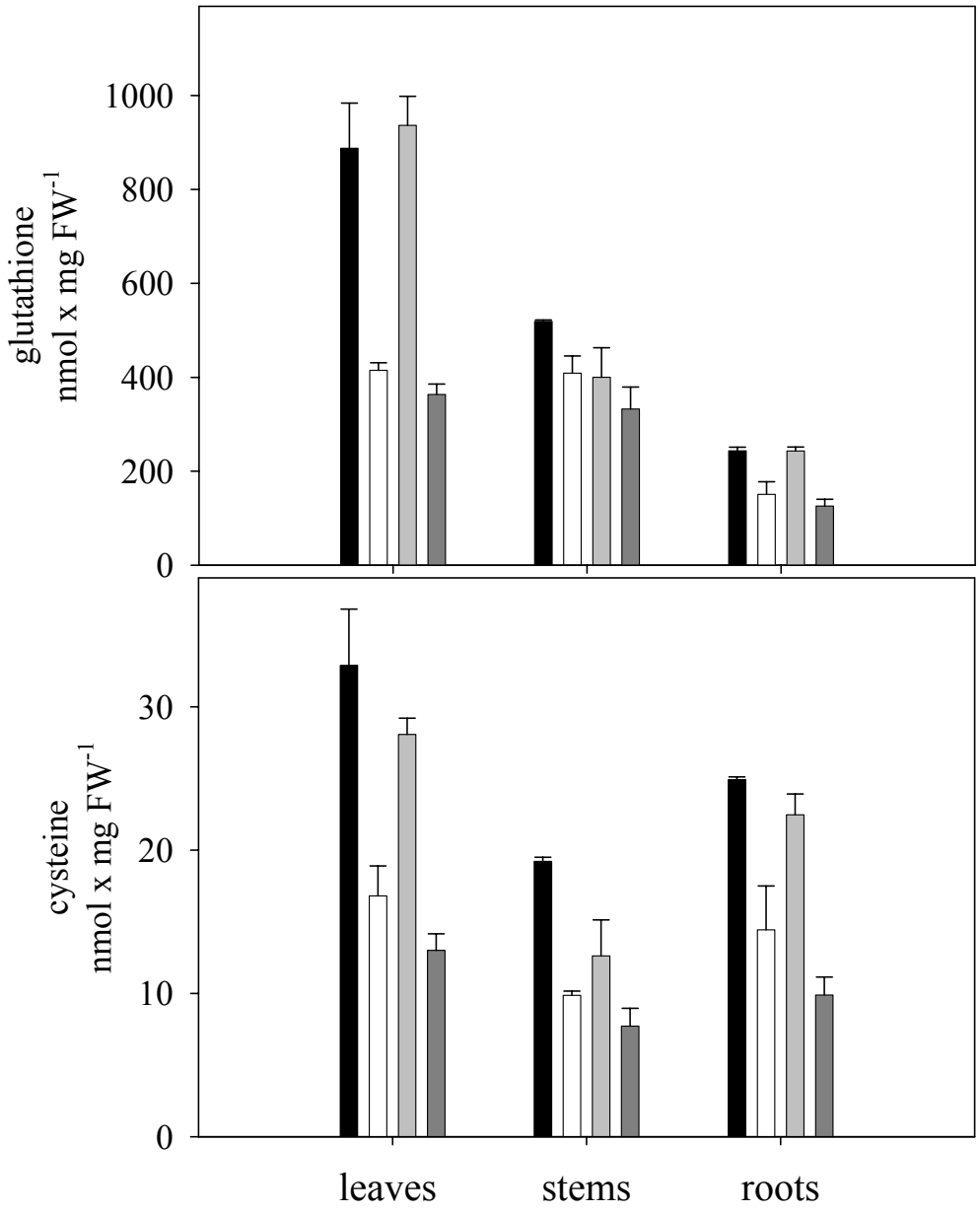


Fig 5: Measurement of the cysteine and glutathione contents in leaves, stems and roots of *B. napus* lines Lion and genotype 15157 via HPLC. The black and grey bars show the control plants compared with plants under sulfur starvation. The thiol concentration is calculated per one mg fresh weight (FW) of the plant material.

Thiol concentration and enzyme activity in* Brassica napus *plants under sulfur starvation

Measurements of the sulfate concentration in different organs in the plants grown under sulfur starvation have shown that the sulfate content was reduced extremly in roots. Samples measured from leaves and stem have also shown reduced sulfate contents (data not shown). Subsequent measurements of the thiols cysteine and GSH (Fig. 5) also show a decrease of about 50% in comparison to plants grown in minus sulfate medium.

Focusing on the cysteine desulfhydrase enzyme activity in Lion plants (Fig. 6) the activity measured in leaves was the lowest independent from the sulfur status of the plant. Therefore, the measurements of the D- and L-cysteine desulfhydrases activity show significant differences in stems and roots. The D-cysteine desulfhydrase enzyme activity is almost half of the L-cysteine desulfhydrase activity in stems and roots. The L-cysteine desulfhydrase activity is nearly four times higher in stems than in leaves.

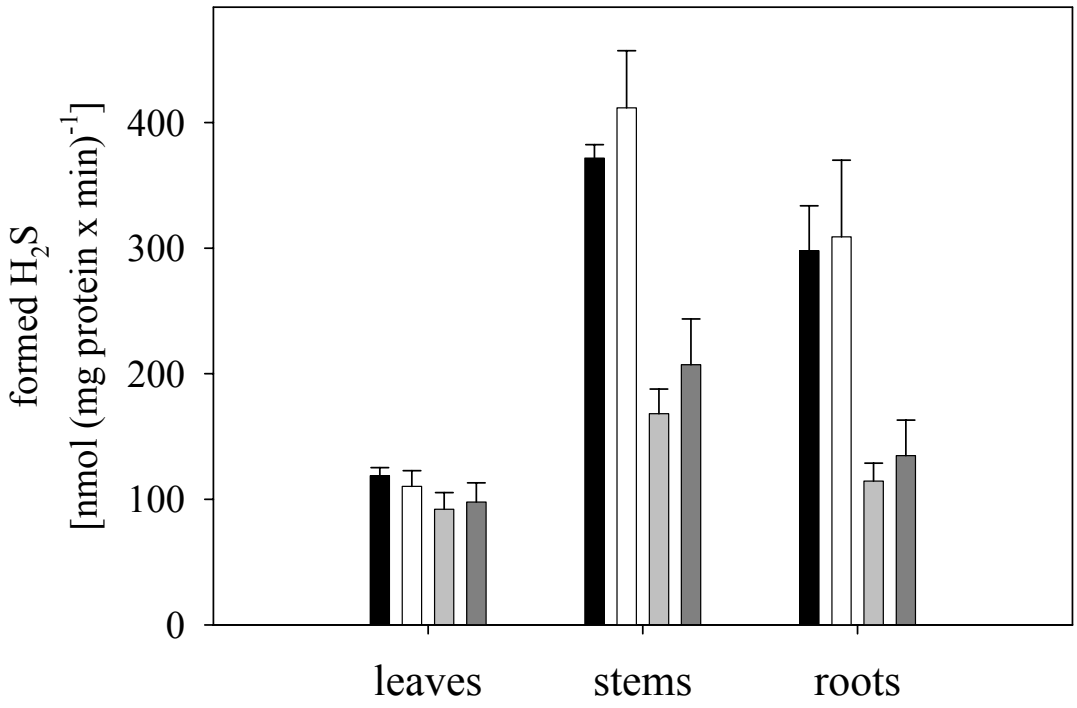


Fig 6: Release of H_2S catalysed from D- and L-cysteine desulfhydrases from crude *B. napus* plant extracts. The plants have been grown in 25% Hoagland medium and half of the plants have been transferred for five days in medium without sulfur. The black and grey bars show the L- and D-cysteine desulfhydrase enzyme activity in control plants. The enzyme activity was measured in leaves, stems and roots.

Discussion

Impact of different pathogens on different lines of winter oilseed rape

In Burandt et al. (2001a) the total sulfur content has been measured in the different winter oilseed rape lines. Proceeding from these results experiments have been started in humid chambers (Fig. 2). The experiments carried out by Burandt et al. (2001a) have shown that with higher total sulfur content the plants show less infestation with *V. dahliae*. Interestingly, the lines with the highest and the lowest sulfur content showed after 16 days the highest infection with the fungus. This rises up the question if the plants show the same sulfur content Burandt et al. (2001a) were able to measure or if the sulfur content is different.

Infection experiments with *B. napus* lines 1, 4, 6, and 7 with three different bacteria focused on the enzyme activity. The results shown in Fig. 1 immediately reflect the differences between infiltrated and control plants. In line 4 the highest H_2S formation could be observed after inoculation with *P. syringae* pv. *maculicola*. However, the results between the different lines in the desulfhydrase activity show slight differences to each other, but interestingly in the capacity to form cysteine *P. syringae* pv. *tabaci* instead of *P. syringae* pv. *maculicola* shows the highest influence on the plants. Plants infected with *P. syringae* pv. *maculicola* show the lowest activity. Focusing on lines 1 and 4 the situation observed from Burandt et al. (2001a) could be proved true. Proceed from the results from Burandt et al. (2001a) the higher the sulfur content in the plants was the lower the L-cysteine desulfhydrase enzyme activity was and the higher the OAS-TL enzyme activity was. This result might indicate that the hypothesis of SIR is true. Line 1 with the highest sulfur content (Burandt et al. 2001a) shows the lowest H_2S releasing enzyme activity. The results are also compatible with results from field experiments (Bloem et al. 2004) where the *B. napus* plants have shown an increase of the thiol concentration and the L-cysteine desulfhydrase enzyme activity after a *P. brassicae* attack.

Research for desulfhydrases in Brassica napus *influenced by sulfur starvation*

Using studies of Blake-Kalff et al. (1998) as background where the physiological and molecular effects of sulfur nutrition have been observed, studies under equal conditions have been done with regard to the influence on CDes.

Unsatisfactory expression analysis results done with heterologous CDes probes from *Arabidopsis thaliana* led us to search for homologous sequences in *B. napus*. Sequences found for *B. napus* were compared with protein sequences from rice and *Arabidopsis* (Fig. 3). Sequences from *B. napus* show high identity and similarity with *Arabidopsis* sequences. This

implicates the idea that the functions might be the same as in *Arabidopsis*. Buchner et al. (2004a) described this hypothesis for *B. oleracea* sulfate transporters. Only the low similarity and identity on protein level of the BnNFS2 protein to all other NFS2 sequences pose the question of the function and also for the localisation. But also a second *BnNFS1* sequence led to questions. Because of the not fully sequenced *B. napus* genome it is impossible to clarify the question about a second NFS1 in oilseed rape and its function at the moment.

Expression studies in different organs of oilseed rape (Fig. 4) prove to be very promising. Three of four studied desulfhydrases show higher mRNA accumulation in stems. Accumulation of elemental sulfur in veines and vascular tissues of resistant plants was studied previously (Cooper et al. 1996; Cooper et al. 2004; Williams et al. 2004). These results might indicate involvement of CDes in the synthesis of elemental sulfur. A heterologous expression in *E. coli* and the subsequent enzyme analysis should clarify whether the isolated *B. napus* sequences show the same enzyme activity observed for *Arabidopsis* CDes. The results from this experiments described above are only steady-state results in a sulfur starvation experiment. Experiments for the future with pathogens should follow and also the method of *in situ* hybridisation should be used to reveal perhaps an involvement of CDes in the protection of plants in the vascular tissue discussed from Williams et al. (2004).

In this experiment the cysteine and GSH concentration decreased from leaves to roots via stems (Fig. 5). The sulfate concentration also decreased during sulfur starvation. In *Arabidopsis* the same phenomenon has been observed for sulfate and GSH (Hirai et al. 2003). From results observed in *B. napus* plants it was concluded that the GSH and glucosinolate content in the plants are not major sources of sulfur during sulfur deficiency (Blake-Kalff et al. 1998). Support on this meaning it would indicate that under sulfur starvation these compounds are not adequate for a fast and efficient answer to pathogen attack although they are antimicrobial itself ((Rausch & Wachter 2005).

An increase of reduced GSH has been observed in response to biotic and abiotic stress (Dhindsa 1991; May et al. 1996; 1998). Leaves reflect the one of the highest surface of a plant and the highest chloroplast concentration, so that might explain why the cysteine and reduced GSH content is highest in leaves. Does reduced GSH function as signal molecule and lead to an accumulation of *CDes* mRNA in stems? It is only possible to keep in mind that the higher the sulfur content in plants, the higher the synthesis of sulfur containing compounds and H_2S release is.

Because of the detection of D-amino acids in nearly all plants found in low percentage range of 0,5 to 3% compared to their L-enantiomers (Brückner & Westerhauser 2003), and the

identification of a D-cysteine desulfhydrase in *Arabidopsis* (Riemenschneider et al. 2005a), D- and L-cysteine were used as substrates (Fig. 6). The H_2S release from *B. napus* plants grown under sulfur starvation does not show any significant differences in the enzyme activity in leaves. Sulfur starvation has no influence on the D- or L-cysteine desulfhydrase enzyme activity, the concentration of released H_2S differs only in stems and roots between the two enantiomers as substrate. Remembering the results of the Northern blot analysis the results are coherent. Also higher *BnL-CDesI* mRNA accumulation in roots could be explained with the idea, that desulfhydrases act with H_2S release against pathogen attack and for example *V. dahliae* is well established as root fungus (Paul 1992). The fungus attack takes place from the roots via the vascular tissue to the leaves.

Now that we know more about *B. napus* desulfhydrase sequences and desulfhydrase expression, we are able to focus on experiments with respect to effects of pathogen attack on CDes in different lines of *B. napus*. It would be very interesting to know if the thiol content changes during infection and if CDes play a role therein, because of producing e.g. elemental sulfur.

Chapter 7

Isolation and characterization of a D-cysteine desulfhydrase protein from *Arabidopsis thaliana*

In several organisms D-cysteine desulfhydrase (D-CDes) activity (EC 4.1.99.4) was measured; this enzyme decomposes D-cysteine into pyruvate, H_2S, and NH_3. A gene encoding a putative D-CDes protein was identified in *Arabidopsis thaliana* (L) Heynh. based on high homology to an *Escherichia coli* protein called YedO that has D-CDes activity. The deduced *Arabidopsis* protein consists of 401 amino acids and has a molecular mass of 43.9 kDa. It contains a pyridoxal-5'-phosphate binding site. The purified recombinant mature protein had a K_m for D-cysteine of 0.25 mM. Only D-cysteine but not L-cysteine was converted by D-CDes to pyruvate, H_2S, and NH_3. The activity was inhibited by aminooxy acetic acid and hydroxylamine, inhibitors specific for pyridoxal-5'-phosphate-dependent proteins, at low micromolar concentrations. The protein did not exhibit 1-aminocyclopropane-1-carboxylate deaminase activity (EC 3.5.99.7) as homologous bacterial proteins. Western blot analysis of isolated organelles and localization studies using fusion constructs with the green fluorescent protein indicated an intracellular localization of the nuclear encoded D-CDes protein in the mitochondria.

D-CDes RNA levels increased with proceeding development of *Arabidopsis* but decreased in senescent plants; D-CDes protein levels remained almost unchanged in the same plants whereas specific D-CDes activity was highest in senescent plants. In plants grown in a 12-h light/12-h dark rhythm D-CDes RNA levels were highest in the dark, whereas protein levels and enzyme activity were lower in the dark period than in the light indicating post-translational regulation. Plants grown under low sulfate concentration showed an accumulation of D-CDes RNA and increased protein levels, the D-CDes activity was almost unchanged. Putative *in vivo* functions of the *Arabidopsis* D-CDes protein are discussed.

Introduction

It is well documented that, in general, amino acids are used in the L-form, and enzymes involved in their metabolism are stereospecific for the L-enantiomers. However, D-amino

acids are widely distributed in living organisms (Friedman 1999). Examples of the natural occurrence of D-amino acids include D-amino acid-containing natural peptide toxins (Sivonen et al. 1992), antibacterial diastereomeric peptides (Oren et al. 1997), and the presence of D-amino acids at high concentrations in human brain (Schell et al. 1997). In plants D-amino acids were detected in gymnosperms as well as mono- and dicotyledonous angiosperms of major plant families. Free D-amino acids in the low percentage range of 0.5–3% relative to their L-enantiomers are principle constituents of plants (Brückner & Westerhauser 2003). The functions of D-amino acids and their metabolism are largely unknown. Various pyridoxal-5'-phosphate (PLP)-dependent enzymes that catalyse elimination and replacement reactions of amino acids have been purified and characterized (Mehta & Christen 2000). However, most act specifically on L-amino acids. Only a few PLP enzymes that act on D-amino acids have been found such as D-serine dehydratase (Dowhan & Snell 1970), 3-chloroD-alanine chloride-lyase (Nagasawa et al. 1982), and D-cysteine desulfhydrase (D-CDes) (Nagasawa et al. 1985; Nagasawa et al. 1988; Schmidt 1982). The *Escherichia coli* D-CDes (EC 4.1.99.4) is capable of catalysing the transformation of D-cysteine into pyruvate, H_2S, and NH_3 (Nagasawa et al. 1985; Nagasawa et al. 1988). A similar activity was detected in several plant species, such as *Spinacia oleracea*, *Chlorella fusca*, *Cucurbita pepo*, *Cucumis sativus* and in suspension cultures of *Nicotiana tabacum* (Schmidt 1982; Schmidt & Erdle 1983; Rennenberg 1983a; Rennenberg et al. 1987). In all publications cited, the D-CDes activity could be clearly separated from L-CDes activity by demonstrating different pH optima for the enzyme activity (Schmidt 1982), different sensitivity to inhibitors (Rennenberg et al. 1987), and different localization in the cell (Rennenberg et al. 1987). Both CDes protein fractions were separated by conventional column chromatography, however, because of low protein stability and low yields neither of the proteins could be purified to homogeneity from plant material (Schmidt 1982; Schmidt & Erdle 1983).

The D-CDes protein from *E. coli* is a PLP-containing enzyme. It catalyses the α,β-elimination reaction of D-cysteine and of several D-cysteine derivatives, and also the formation of D-cysteine or D-cysteine-related amino acids from β-chloro-D-alanine in the presence of various thiols or from *O*-acetyl-D-serine and H_2S (Nagasawa et al. 1985; Nagasawa et al. 1988). The physiological role of bacterial D-CDes is unknown. Studies indicated that *E. coli* growth is impaired in the presence if micromolar amounts of D-cysteine (Soutourina et al. 2000). To assess the role of D-CDes in adaptation to D-cysteine, a gene was cloned from *E. coli* corresponding to the ORF *yedO* at 43.03 min on the genetic map of *E. coli* (Soutourina et al. 2001) (protein accession number D64955). The amino acid sequence deduced from this gene

is homologous to those of several bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminases. However, the *E. coli* YedO protein did not use ACC as substrate, but exhibited D-CDes activity. *YedO* mutants exhibited hypersensitivity or resistance, respectively, to the presence of D-cysteine in the culture medium. It was suggested that D-cysteine exerts its toxicity through an inhibition of threonine deaminase. On the other hand, the presence of the *yedO* gene stimulates cell growth in the presence of D-cysteine as sole sulfur source because the bacterium can utilize H_2S released from D-cysteine as sulfur source. Consequently, the *yedO* expression was induced by sulfur limitation (Soutourina et al. 2001).

In the *Arabidopsis* genome, a gene homologous to *yedO* has been identified (Soutourina et al. 2001) (At1g48420). To date ACC deaminase activity has not been demonstrated for plants. Therefore the tentative annotation as an ACC deaminase is probably not correct and the deduced protein might be a good candidate for the first D-CDes enzyme in higher plants of which the sequence is known. The putative D-CDes encoding cDNA was amplified by RT/PCR from *Arabidopsis*, the protein was expressed in *E. coli*, and the purified protein was analysed enzymatically. It was shown to exhibit D-CDes activity with the products pyruvate, H_2S, and NH_3. The nuclear-encoded protein was transported into mitochondria. Expression analysis revealed higher D-CDes mRNA and protein levels in older plants, during the light phase in a diurnal light/dark rhythm and under sulfate limitation.

Experimental procedures

Growth and harvest of plants

Seeds of *Arabidopsis thaliana* (L) Heynh., ecotype C24, were originally obtained from the Arabidopsis stock centre at the Ohio State University. Seeds were germinated on substrate TKS1 and after 2 weeks the plants were transplanted into pots (diameter 7 cm) in TKS2 (Floragard, Oldenburg, Germany). Plants were grown in the greenhouse in a 16-h light/8-h dark rhythm at a temperature of 23°C/21°C. When necessary, additional light was switched on for 16 h per day to obtain a constant quantum fluence rate of 300 $\mu mol*m^{-2}*s^{-1}$ (sodium vapour lamps, SON-T Agro 400, Philips, Hamburg, Germany).

To investigate natural senescence, *Arabidopsis* plants were grown in the greenhouse for up to 6 weeks counted from transfer into pots, and the parts above ground were cut every week. The oldest leaves were comparable to the S3 stage as defined (Lohmann et al. 1994). The influence of light and darkness on expression and activity were investigated in 4-week-old plants grown in a 12-h light/12-h dark cycle in a growth chamber at a quantum fluence rate of 50 $\mu mol* m^{-2} *s^{-1}$ (TLD 58 W/33, Philips), and a constant temperature of 22°C. To follow one

complete diurnal cycle, plant parts above ground were harvested every 4 h for 1.5 days starting 1 h after the onset of light. To investigate the influence of high and low sulfate concentrations in the growing medium, *Arabidopsis* seeds were germinated under sterile conditions and grown for a further 18 days in a hydroponic culture system under sterile conditions (Schlesinger et al. 2003) in MS medium prepared according to (Murashige & Skoog 1962) containing modified sulfate concentrations of 500 µm (high) and 50 µm (low), respectively.

Cloning procedures

RNA was extracted from cut leaves of 3-week-old *Arabidopsis* plants, ecotype C24, and transcribed into cDNA by RT/PCR according to manufacturer's instruction (Super-ScriptII RNase H– reverse transcriptase; Invitrogen, Karlsruhe, Germany). To obtain an expression clone the following primer pair was used to amplify a 1203-bp sequence encoding the full-length D-CDes protein: primer 102 (5'-CGGATCCAGAGGACGAAGCTTGACA-3') extended by a *BamHI* restriction site and primer 103 (5'-CTGCAGGAACATTTTCCCAACACC-3') extended by a *PstI* restriction site. Primer 308 (5'-GGATCCTCTGCAA CATCCGTA-3') extended by a *BamHI* restriction site and primer 103 were used to amplify a 1143-bp sequence encoding the putative mature D-CDes protein. The following primer pair was used for the amplification of a 1203-bp DNA fragment for cloning into a vector containing the sequence encoding the GFP: primer 238 (5'-CCATGGGA GGACGAAGCTTGACA-3') extended by an *NcoI* restriction site and primer 239 (5'-AGATCTGAACATTTTCCCAACACC-3') extended by a *BglII* restriction site.

The PCR tubes contained 0.2 mM dNTPs (Roth, Karlsruhe, Germany), 0.4 µM of each primer (MWG, Ebersberg, Germany), 1 mM $MgCl_2$ (final concentration, respectively), 0.75 µl RedTaq DNA-Polymerase (Sigma, Taufkirchen, Germany), and ~1 µg template DNA in a final volume of 50 µl. Before starting the first PCR cycle, the DNA was denatured for 180 s at 94°C followed by 28 PCR cycles conducted for 45 s at 94°C, 45 s at 52°C, and 45 s at 72°C. The process was finished with an elongation phase of 420 s at 72°C. The amplified PCR fragments were ligated either into the expression vector pQE-30 (Qiagen, Hilden, Germany) or into pBSK-based enhanced GFP-containing vectors (Bauer et al., 2004) to obtain either GFP fusions with the 5' end of the GFP coding sequence (pGFP-N/D-CDes) or with the 3' end (pGFP-C/D-CDes) and were introduced into the *E. coli* strain XL1-blue.

Expression and purification of the D-CDes protein

The putative D-CDes protein was expressed in *E. coli* according to the following protocol: after growth of the respective *E. coli* cultures at 37°C to OD_{600} to 0.6 in Luria–Bertani medium (10 g*l^{-1} tryptone, 5 g*l^{-1} yeast extract, 10 g*l^{-1} NaCl) (Roth, Karlsruhe, Germany) containing ampicillin (100 µg*ml^{-1}) (AppliChem, Darmstadt, Germany) induction was carried out for 2 h with 1 mM (final concentration) of isopropyl thio-β-D-galactoside (IPTG) (AppliChem). Cell lysis was obtained by adding lysozyme (final concentration 1 mg* ml^{-1}) (Roth) and vigorous homogenizing using a glass homogenizer and a pestel. The recombinant protein was purified under nondenaturing conditions by affinity chromatography with the nickel resin according to manufacturer's instructions (Qiagen) and dialysed overnight against dialysis buffer (20 mM Tris/HCl pH 8.0) at 4°C. The purity of the protein preparations was controlled by SDS/PAGE (Laemmli 1970) and subsequent staining with Coomassie blue- or silver stain (Heukeshoven & Dernick 1988).

For antibody production purified D-CDes protein (400 µg) was subjected to preparative SDS/PAGE. Gel slices containing the D-CDes protein were ground in liquid nitrogen. Proteins were extracted in 2 ml phosphate buffer. To aliquots of 100 µg protein in 500 µl phosphate buffer 500 µl Freund's complete adjuvant was added to completely denature the proteins and the mixture was used for two subsequent immunizations of two rabbits according to standard procedures. In western blot trials using D-CDes recombinant protein and *Arabidopsis* leaf extracts one of the two sera reacted with a single protein; it was used for further experiments.

Transient expression of the GFP fusion constructs in* Arabidopsis *protoplasts

The younger rosette leaves of 3-week-old *Arabidopsis* plants grown in the greenhouse as described above were used for the preparation of protoplasts essentially as described (Damm et al. 1989; Sheen 1995; Abel & Theologis 1998). About 40 leaves were cut in 1-mm strips with sharp razor blades and put in 6 ml of medium I [1% (w/v) cellulase Onozuka R-10, 0.25% (w/v) macerozyme R-10 (Yakult Honsha, Tokyo, Japan), 0.4 M mannitol, 20 mM KCl, 20 mM Mes/KOH pH 5.7, 10 mM $CaCl_2$, 5 mM 2-mercaptoethanol, 0.1% (w/v) BSA]. After application of a vacuum for 20 min the leaves were incubated while shaking at 40 r.p.m. for 60 min at room temperature. The suspension was filtered through a 75-µm nylon net, the filtrate was distributed into six 2-ml tubes and centrifuged for 2 min at 95 g and 4°C. The pellet was washed twice with 500 µl medium II (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM Mes/KOH, pH 5.7) and finally incubated for 30 min on ice in medium II. After

centrifugation for 2 min at 95 g and 4°C the pellet was carefully resuspended in 150µl medium III (0.4 M mannitol, 15 mM $MgCl_2$, 4 mM Mes/KOH, pH 5.7). For the transformation 100 µl of the protoplast suspension was carefully mixed with 15 µg column-purified plasmid DNA (Plasmid Midi Kit, Qiagen) and 110 µl medium IV (4 g PEG 4000, 3 ml H_2O, 2.5 ml 0.8 M mannitol, 1 ml 1 M $CaCl_2$) and incubated for 30 min at 23°C. To remove the PEG 1 ml medium II was added, centrifuged for 2 min at 95 g and 4°C, and finally the pellet was resuspended in 50 µl medium II and incubated overnight at 23°C. The transiently transformed protoplasts were analysed by fluorescence microscopy (Axioskop Zeiss, Jena, Germany) with the following filter set: excitation 450–490 nm (filter BP 450–490) and emission 520 nm (filter LP520) for GFP. All images were prepared with Corel photo paint 10.

RNA extraction and Northern blot analysis

Total RNA was extracted essentially as described (Chomczynski & Sacchi 1987). RNA samples (20 µg) were separated on 1% denaturing agarose/formaldehyde gels. Equal loading was controlled by staining the gels with ethidium bromide. After transfer of RNA onto nylon membranes, the filters were hybridised with digoxigenin (DIG)-labelled cDNA probes obtained by PCR. To label the D-CDes cDNA the sequence-specific primers 102 and 103 were used in the PCR DIG probe synthesis kit (Roche, Mannheim, Germany). Colorimetric or chemiluminescent detection methods with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) or with CDP-Star (Roche) as substrates for alkaline phosphatase were applied.

SDS/PAGE of plant samples and Western blotting

For the determination of D-CDes protein steady-state levels in *Arabidopsis* plants, 100 mg plant material was ground with a mortar and pestle in liquid nitrogen to a fine powder. Sample buffer [500 µl; 56 mM Na_2CO_3, 56 mM DTT, 2% (w/v) SDS, 12% (w/v) sucrose, 2 mM EDTA] was added, samples were heated at 95°C for 15 min and cell debris was removed by centrifugation. Ten micrograms of the protein extracts was subjected to denaturing SDS/PAGE according to Laemmli (1970) and blotted (Sambrook et al. 1989). A colorimetric detection method using NBT and BCIP or the ECL Western blotting analysis system (Amersham Biosciences, Freiburg, Germany) was applied.

Organelle fractionation

Mitochondria were prepared from *Arabidopsis* suspension cultures (May & Leaver, 1993; Werhahn et al., 2001) and chloroplasts were isolated from green *Arabidopsis* plants (Jensen & Bassham, 1966). The purity and the intactness of mitochondrial fractions chloroplasts were analysed as described (Hausmann et al., 2003; Leegood & Malkin, 1986).

Preparation of protein extracts for the determination of enzyme activity

The recombinant D-CDes protein was purified as described above and adjusted with dialysing buffer to a protein concentration of 10 $\mu g*ml^{-1}$. To obtain crude *E. coli* extracts, cultures of the *E. coli* strain XL1-blue transformed with the pQE-30 vector without or with the D-CDes encoding insert were grown to OD_{600} to 0.6, 1 mM IPTG was added, and growth was continued for 2 h. Soluble protein extracts were obtained as described for the purification procedure by affinity chromatography. The protein concentration was adjusted to 10 $\mu g*ml^{-1}$ with dialysis buffer. Plant material was ground with a mortar and pestle in liquid nitrogen and the soluble proteins were extracted by adding 20 mM Tris/HCl pH 8.0, in a ratio of 1:10 (100 mg plant material/ 900 µl buffer). After centrifugation (16.060 g for 20 min at 4°C) the protein content of the supernatant was adjusted to 100 $\mu g*ml^{-1}$ to obtain equal amounts of protein in each assay sample (10 µg).

Enzyme activity measurements

Measurement of H_2S formation

The D-CDes activity was measured by the release of H_2S from D-cysteine. The assay contained in a total volume of 1 ml 100 mM Tris/HCl pH 8.0, various amounts of different protein extracts, and 1 mM DTT. The reaction was started by the addition of 1 mM D-cysteine, incubated for 15 min at 37°C, and terminated by adding 100 µl of 30 mM $FeCl_3$ dissolved in 1.2 N HCl and 100 µl 20 mM N,N-dimethyl-p-phenylenediamine dihydrochloride dissolved in 7.2 N HCl (Siegel 1965). The formation of methylene blue was determined at 670 nm in a spectrophotometer. Solutions with different concentrations of Na_2S were prepared, treated in the same way as the assay samples, and were used for the quantification of the enzymatically formed H_2S.

Measurement of pyruvate formation

The activity of the purified D-CDes enzyme was followed through the measurement of the amount of pyruvate formed from D-cysteine by using a spectrophotometric method with lactate dehydrogenase and NADH as described (Nagasawa et al. 1985). The reaction was

performed at 37°C in 1-ml cuvettes containing 50 mM potassium phosphate buffer (pH 8.0), 0.13 mM NADH, 5 U rabbit muscle lactate dehydrogenase, 1 mM of the respective substrate (D-cysteine, L-cysteine, or β-chloro-D-alanine), and catalytic amounts of enzyme (10 µg). The reaction was started by adding the substrate solution. Consumption of NADH and the resulting decrease in absorption at 340 nm were monitored. The amount of pyruvate produced was calculated using the molar absorption coefficient of 6220 M^{-1} for NADH. NH_3 was determined using Nessler's reagent as described (Yuen & Pollard 1954). To the assay volume of 1 ml 100 µl 3 N NaOH and 10 µl Nessler's reagent (Sigma) were added and the absorbance was directly measurement at 400 nm. The NH_3 was quantified by preparing a standard curve with ammoniumnitrate.

Measurement of ACC deaminase activity

Colorimetric formation of 2-oxobutyrate, based on the reaction of this compound with 2,4-dinitrophenylhydrazine was used to obtain evidence for a possible conversion of ACC into 2-oxobutyrate as described in (Jia et al. 1999) using 1 µg recombinant protein. OAS-TL and β-cyanoalanine synthase activities were determined as described (Burandt et al. 2002).

Other procedures

Protein contents were determined according to (Bradford 1976) using BSA as a protein standard. The DNA and amino acid sequence analyses and prediction of the molecular masses were performed with the programs MAPDRAW and PROTEAN in DNASTAR (Lasergene, DNASTAR, Madison, WI, USA). For the identification of protein domains several programs in http://www.expasy.ch were used. For the prediction of the protein localization different programs were applied (IPSORT, MITOPROT, PSORT, PREDATOR, and TARGETP, http://www.expasy.ch/tools). The multiple sequence alignment was performed using CLUSTALW (http://www.ebi.ac.uk/clustalw). Statistical analysis was performed using the Student's *t*-test (sigmaplot for windows version 7.0). The K_m values were calculated from the nonlinear Michaelis–Menten plot using an enzyme kinetic program (SIGMAPLOT 7.0).

Results

In silico characterization and isolation of the Arabidopsis *protein homologous to YedO from* E. coli

The existence of D-CDes activity was demonstrated in different plant species a long time ago and it could be shown that at least part of the activity was PLP dependent (Schmidt & Erdle 1983; Rennenberg et al. 1987; Schmidt 1987). However, the respective encoding gene(s) had not been identified in any plant species because the putative D-CDes protein from spinach could not be purified to sufficient homogeneity for amino acid sequencing (data not shown). Recently, a protein with D-CDes activity and its respective gene, called *yedO*, were isolated from *E. coli* (Soutourina et al. 2001). Consequently, the sequenced *Arabidopsis* genome (The *Arabidopsis* genome initiative 2000) was screened for homologues to the *E. coli yedO* gene. The highest identities at both the nucleotide and the amino acid levels revealed a sequence that had been annotated based on sequence homologies to several bacterial proteins such as ACC deaminase (EC 3.5.99.7), an enzyme activity not identified in plants to date. The putative D-CDes encoding *Arabidopsis* gene is located on chromosome 1 (At1g48420, DNA ID NM_103738, protein ID NP_175275). The corresponding EST clone VBVEE07 from *Arabidopsis*, ecotype Columbia (available from the *Arabidopsis* stock Resource center, DNA Stock Center, The Ohio State University) was not complete at the 5' end. The complete coding region of 1203 bp was obtained by RT/PCR from RNA isolated from 3-week-old *Arabidopsis* plants. The respective D-CDes protein consists of 401 amino acids including the initiator methionine and excluding the terminating amino acid. The protein has a predicted molecular mass of 43.9 kDa and a pI of 7.2. It contains relatively high amounts of the sulfur amino acids cysteine (four residues) and methionine (10 residues). According to several programs predicting the intracellular localization of proteins in the cell (http://www.expasy.ch/tools), the protein might possess an N-terminal extension (in PSORT, a probability of 0.908 for mitochondria; PREDATOR, mitochondrial score of 0.965; MITOPROT, 0.9547 probability of export to mitochondria). In PSORT a protease cleavage site between amino acids 19 and 20 counting from the start methionine was predicted, indicating a presequence of 19 amino acids. The mature protein would have a molecular mass of 41.7 kDa and a pI of 6.34.

The YedO protein from *E. coli* and the D-CDes from *Arabidopsis* showed an overall identity of 36% and a similarity of 50%. The BLASTP program in its default positions was used to identify eukaryotic protein sequences revealing sequence similarities to the *Arabidopsis* D-CDes protein. The resulting phylogenetic tree including the YedO protein sequence is shown

(Fig. 1). Two proteins closely related to *Arabidopsis* D-CDes were detected in the plant species *Oryza sativa* and *Betula pendula*. The YedO protein from *E. coli* showed higher similarities to the plant D-CDes protein than to related proteins from several yeast species (for clarity only representative sequences from three species are shown). The respective protein from *Hansenula saturnus* was already crystallized and a model of its 3D structure determined (Yao et al. 2000). Interestingly, both *Arabidopsis* and *Oryza* contain a second protein revealing a lower sequence similarity to the true D-CDes proteins. Their function is unknown so far.

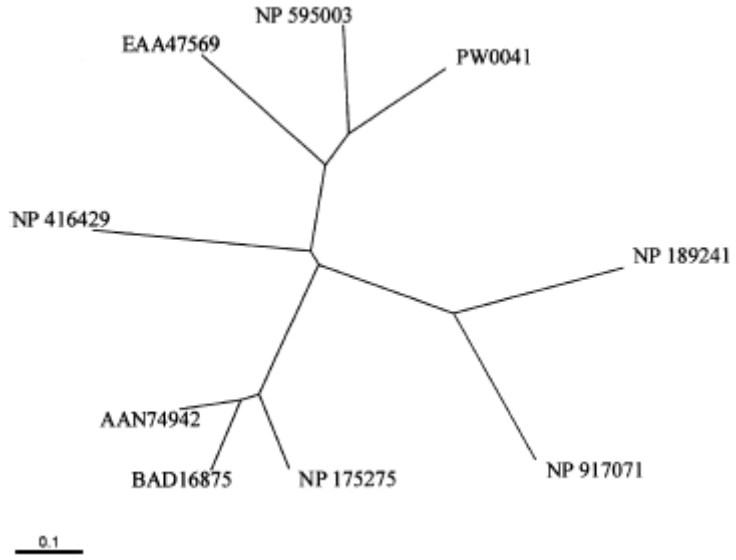


Fig. 1: Phylogenetic tree of eukaryotic D-CDes sequences and the *E. coli* YedO sequence. The D-CDes protein sequence from *Arabidopsis* was used in BLASTP to identify eukaryotic protein sequences revealing the highest similarities. The species and the respective protein accession numbers are given: NP_416429, YedO, *E. coli*; NP_175275, D-CDes, *Arabidopsis thaliana*; BAD16875, *Oryza sativa*; AAN74942, *Betula pendula*; NP_595003, *Schizosaccharomyces pombe*; EAA47569, *Magnaporthe grisea*; PW0041, *Hansenula saturnus*; NP_189241, *Arabidopsis thaliana* (lower similarity); NP_917071, *Oryza sativa* (lower similarity).

All enzymes aligned belong to the PLP-dependent protein family (PALP, PF00291, http://pfam.wustl.edu/hmmsearch.shtml). Members of this protein family catalyse manifold reactions in the metabolism of amino acids. In addition to the PLP-binding site a number of other PROSITE (http://expasy.hcuge.ch/sprot/prosite.html) patterns and rules were detected in the D-CDes protein sequence, such as N-glycosylation, tyrosine sulfation, phosphorylation,

myristylation, and amidation sites, all of them are characterized by a high probability of occurrence.

Enzyme activity of the recombinant protein

The recombinant *Arabidopsis* D-CDes proteins including and excluding the targeting peptide were expressed in *E. coli* and already 2 h after induction the proteins accumulated up to 5% of the total *E. coli* protein (Fig. 2). The D-CDes proteins were purified by nickel affinity chromatography under native conditions to about 95% homogeneity as demonstrated by loading the purified protein fraction on an SDS-containing gel and subsequent Coomassie- and silver-staining. The Coomassie-stained SDS gel visualizing the purified mature D-CDes protein is shown in Fig. 2. The purified recombinant D-CDes proteins including and excluding the targeting peptide were dialysed overnight against 20 mM Tris/HCl pH 8.0 and used for enzyme assays.

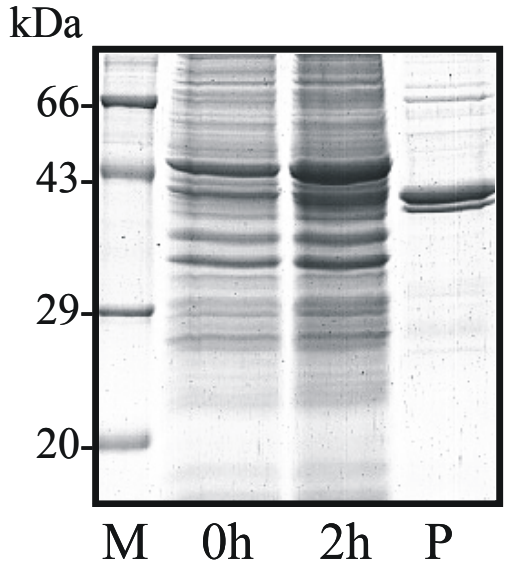


Fig. 2: SDS/PAGE analysis of *E. coli* carrying *Arabidopsis* cDNA encoding the mature D-CDes protein cloned into the pQE-30 expression vector. SDS/PAGE was performed according to Laemmli (1970). Samples were denatured in the presence of 56 mM DTT and 2% SDS, heated for 15 min at 95°C, and centrifuged. Aliquots of the supernatant were loaded onto SDS-containing gels. Lanes described from the left to the right: M, protein marker (Roth); 0 h, protein extract of transformed *E. coli* strain XL1-blue shortly before induction of the culture with IPTG; 2 h, transformed *E. coli* strain XL1-blue protein extract 2 h after induction with IPTG; P, protein purified by Ni^{2+}-affinity chromatography (10 µg). The molecular masses of the marker proteins are given in kDa on the left.

The pH optimum for the D-CDes reaction was determined to pH 8.0, in contrast to L-CDes activity with an optimum of pH 9.0 (Burandt et al. 2002). The purified D-CDes proteins were as heat labile as other proteins as demonstrated by incubation experiments in 100 mM Tris/HCl pH 8.0, for 15 min at elevated temperature and subsequent enzyme activity analysis. They lost activity at 50°C and no activity was left at 60°C. However, the D-CDes protein including the targeting peptide was very sensitive to freezing. One freeze–thaw cycle led to a loss of activity of 75%. Several complex dialysing buffers including glycerol, PLP, dithiothreitol and EDTA did not increase the stability of the protein after freezing. The results are in agreement with earlier stability problems during conventional column purification (Schmidt 1987). The mature D-CDes protein that had been expressed without the targeting peptide was more stable with respect to freezing and was therefore used for most of the enzyme assays.

The K_m value for D-cysteine was determined to 0.25 mM. D-cysteine concentrations higher than 2 mM reduced the enzyme activity by substrate inhibition as observed previously for the E. *coli* protein (Nagasawa et al. 1985). The catalytic constant k_{cat} was determined to 6.00 s^{-1}. The molecular mass for the recombinant protein was calculated excluding the His_6-tag (41.7 kDa). The catalytic efficiency was determined to be 24 $mM^{-1}*s^{-1}$. The enzyme activity using L-cysteine as substrate showed only about 5% of the D-CDes activity indicating a high specificity for D-cysteine.

In previous experiments it was demonstrated that the *E. coli* D-CDes protein catalysed the β-replacement reaction of *O*-acetyl-D-serine with sulfide to form D-cysteine (Nagasawa et al. 1988). Therefore it was tested whether the *Arabidopsis* D-CDes protein exhibits *O*-acetyl-D-serine(thiol)lyase or *O*-acetyl-L-serine(thiol)lyase activity, this was not the case. β-chloro-D-alanine and β-chloro-L-alanine were used in the *O*-acetyl-L-serine(thiol)lyase (OAS-TL) assay instead of *O*-acetyl-L/D-serine and the formation of cysteine was determined; the D-CDes protein did not reveal any activity in this assay. The protein was also tested for β-cyanoalanine synthase activity by using D-cysteine and cyanide as substrates; the D-CDes protein did not show any β-cyanoalanine synthase activity.

Because originally the protein was identified as an ACC deaminase the recombinant D-CDes protein was used to determine this enzyme activity according to Jia et al. (1999). The recombinant protein did not show any ACC deaminase activity. Plant extracts of the soluble protein fraction did not exhibit ACC deaminase activity either.

As mentioned above the D-CDes protein contains a PLP-binding site and was grouped into the PALP family. The absorption spectrum of the purified D-CDes protein determined

between 250 and 470 nm revealed a small shoulder at 412 nm (data not shown), indicating the presence of the cofactor PLP. The ratio A_{280}:A_{412} was ~21.4:1. A molar ratio of PLP (A_{412}) to protein (A_{280}) of 2:1 would suggest that there was one molecule of PLP associated with one protein molecule. The protein preparation was not completely pure as seen in Fig. 2. However, the ratio indicates that not all D-CDes protein molecules contained the PLP cofactor. Addition of pyridoxine and thiamine to the protein expression medium or to the dialysis buffer did not increase the protein/PLP cofactor ratio. To obtain further evidence for the involvement of PLP in the reaction, experiments with specific inhibitors for PLP proteins were performed. The inhibitors aminooxy acetic acid (AOA) and hydroxylamine were applied in the concentration range 10 µM to 5 mM to determine the I_{50} concentration using the purified D-CDes protein in the H_2S-releasing assay. At the higher inhibitor concentrations the activity was completely blocked. The I_{50} for AOA was determined to 30.5 µM and for hydroxylamine to 15.9 µM. The results underline the identification of the D-CDes protein as PLP dependent. In former experiments the I50 for AOA of D-CDes activity in crude homogenates of cucurbit leaves was determined to 100 µM (Schütz et al. 1991).

Additionally, inhibitor experiments were performed in crude extracts of soluble proteins from *Arabidopsis* and *Brassica napus* leaves. The inhibitors AOA and hydroxylamine were used in a concentration range of 50 µM to 50 mM. The D-CDes activity was reduced by AOA to about 45% and by hydroxylamine to about 25% indicating the presence of additional proteins, which are independent from PLP, catalysing D-CDes activity, at least in the Brassicaceae family.

Localization in the cell

Although the *in silico* predictions for the intracellular localization of the D-CDes protein gave consistent results in the three programs mentioned, other programs and scores with the second highest probability gave more diverse results. Thus, the localization of the *Arabidopsis* D-CDes in the cell was investigated experimentally by two different approaches. Total protein extracts and protein extracts from isolated mitochondria and chloroplasts (~15 µg each) were subjected to western blot analysis using a monospecific D-CDes antibody. In total extracts a single band was recognized at ~43 kDa indicating the presence of the full length protein, in mitochondria three bands at about 42, 43 and 44 kDa were detected, while no bands were visible in chloroplast extracts (Fig. 3). One could assume that in mitochondria the unprocessed protein, the mature protein and a post-translationally modified protein might be

present. N-terminal sequencing and analysis of peptides by MS could help to verify this explanation.

For the second method to examine targeting of D-CDes, fusion constructs with pGFP-N or pGFP-C including the D-CDes targeting peptide sequence were introduced into *Arabidopsis* protoplasts, incubated overnight at room temperature, and visualized by fluorescence microscopy (Fig. 4). Bright field images were taken to visualize the protoplast's cell membrane and chloroplasts. The green fluorescence of the pGFP-N/D-CDes fusion construct indicates a localization in mitochondria in agreement with the western blot results (Fig. 4A). When the D-CDes protein was fused with the C terminus of the green fluorescent protein (GFP) in the pGFP-C vector the fusion protein remained in the cytoplasm (Fig. 4C).

Expression studies on the RNA and protein levels and enzyme activities

Arabidopsis plants were grown in the greenhouse for 10–45 days and all plant tissue above ground was used for the analyses. The D-CDes mRNA levels remained almost constant during aging, indicating a constitutive expression (Fig. 5A). The western blot results using the monospecific D-CDes antibody reflected the mRNA results on the protein level (Fig. 5B). The specific D-CDes activity in crude soluble plant extracts increased with increasing age of the plants (Fig. 5C). Either the protein is activated by a post-translational modification or another protein is responsible for the increased enzyme activity in older plants.

Arabidopsis plants were grown in a 12-h light/12-h dark cycle and the parts above ground were harvested every 4 h and frozen in liquid nitrogen. The D-CDes mRNA levels increased at the end of the light period, reached a maximum at the end of the dark phase and decreased at the beginning of the light cycle. The D-CDes gene expression or the stability of the D-CDes mRNA was negatively regulated by light (Fig. 6A). The Western blot results using the D-CDes antibody were not parallel to the D-CDes mRNA levels, the D-CDes steady-state protein levels remained almost constant during the light/ dark cycle (Fig. 6B). However, the specific D-CDes activity in *Arabidopsis* extracts was slightly, but not significantly (Student's *t*-test at $P < 0.01$) reduced in the dark in contrast with the D-CDes transcript levels (Fig. 6C).

Fig. 3: Determination of the subcellular localization by Western blot analysis. Protein extracts were subjected to the western blot procedure using the monospecific anti-D-CDes antibody as primary antibody. Alkaline phosphatase-coupled antirabbit antibody was used as secondary antibody. Lanes from left to right: total protein extract from *Arabidopsis* leaves (TE, 10 µg); total protein extracts of *Arabidopsis* mitochondria isolated form suspension cell cultures (Mi, 2 µg); total protein extracts of *Arabidopsis* chloroplasts isolated from green leaves (Cp, 2 µg). The size of a marker protein is indicated on the right.

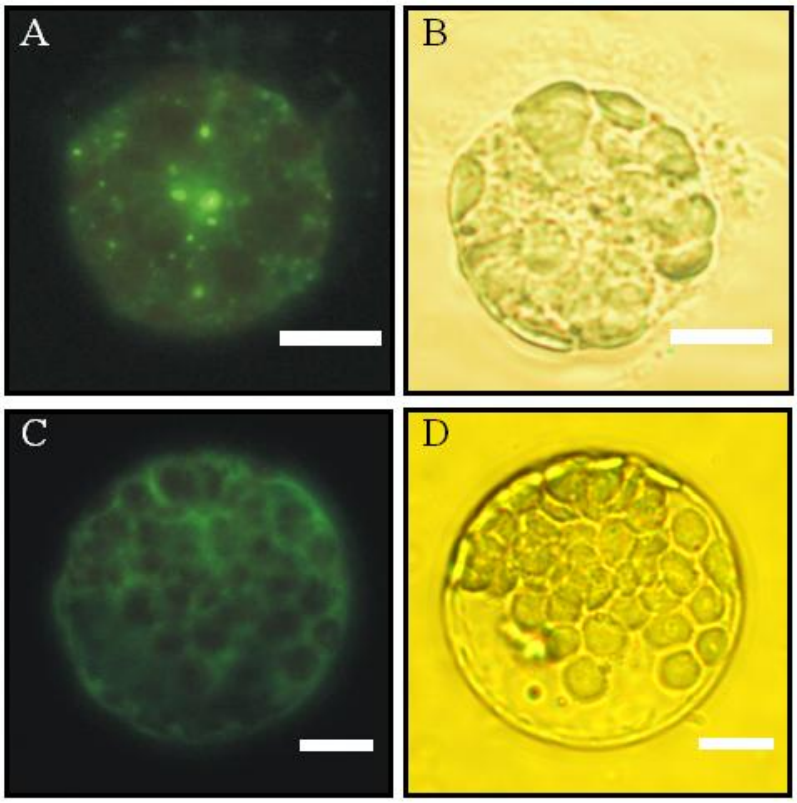


Fig. 4: Intracellular localization of D-CDes GFP fusion constructs. The D-CDes encoding cDNA sequence was ligated in frame into the pGFP-N and the pGFP-C vector, respectively. The fusion constructs were introduced into *A. thaliana* protoplasts. The protoplasts were incubated overnight at room temperature and then analysed with an Axioskop microscope with filter sets optimal for GFP fluorescence (BP 450–490 / LP 520). Fluorescence images of the transformed protoplasts are shown in (A; pGFP-N fusion) and C; pGFP-C fusion). Bright field images of the same protoplasts were made to visualize the protoplast's cell membrane and the chloroplasts (B and D).

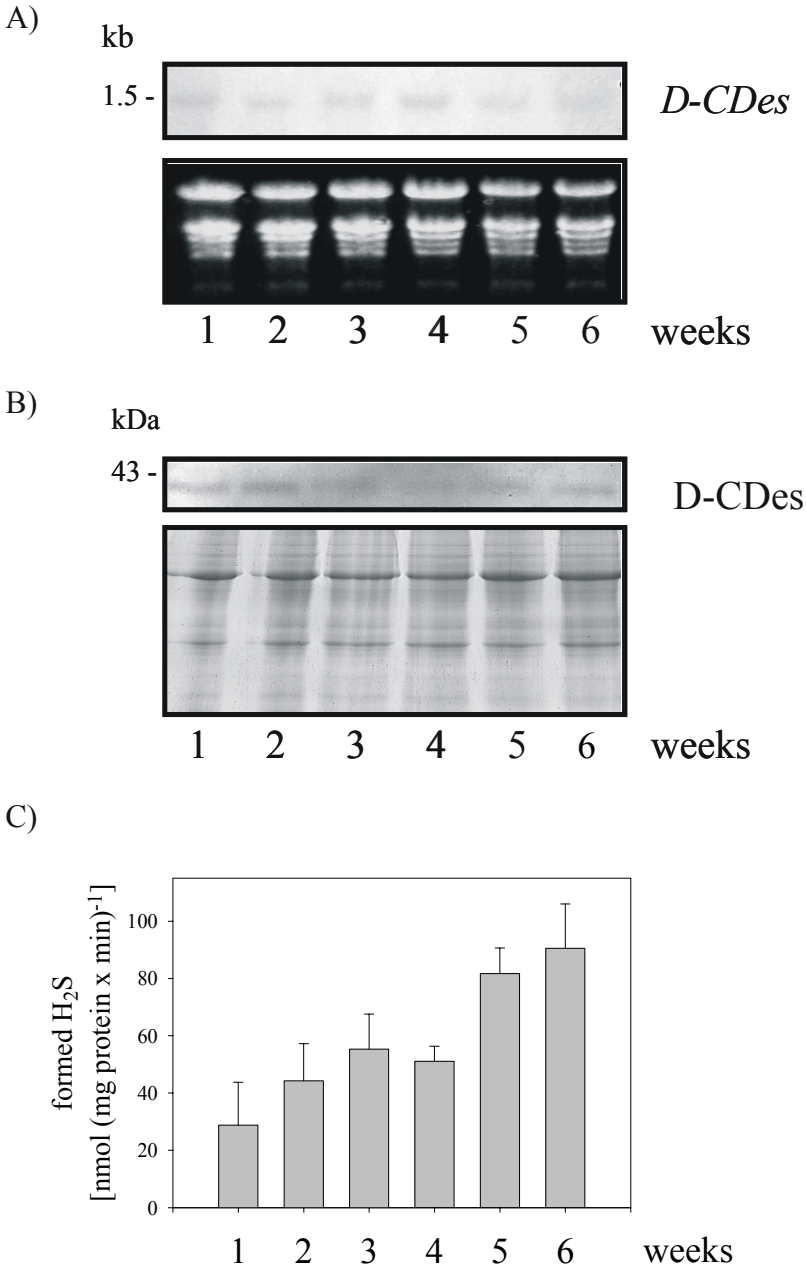


Fig. 5: Expression and activity analyses during aging. *Arabidopsis* plants were grown in the greenhouse for 10–45 days, counted from the transfer into pots, and all plant tissue above ground was used for the analyses. (A) Total RNA was extracted and 20 µg RNA was loaded in each lane and blotted as indicated in Experimental procedures. To prove equal loading of the extracted RNA the ethidium bromide-stained gel is shown at the bottom. D-CDes cDNA was labelled with DIG by PCR. (B) From the same plant material total protein extracts were prepared, separated by SDS/PAGE, and blotted onto nitrocellulose membranes. A monospecific antibody recognizing the D-CDes protein was used for the immunoreaction. The Coomassie blue-stained gel loaded with the same protein samples is shown in the lower panel to demonstrate loading of equal protein amounts. (C) Total extracts of the soluble proteins were prepared from the same plant material and used for the determination of D-CDes enzyme activity. Solutions with different concentrations of Na_2S were used for the quantification of the enzymatically produced H_2S.

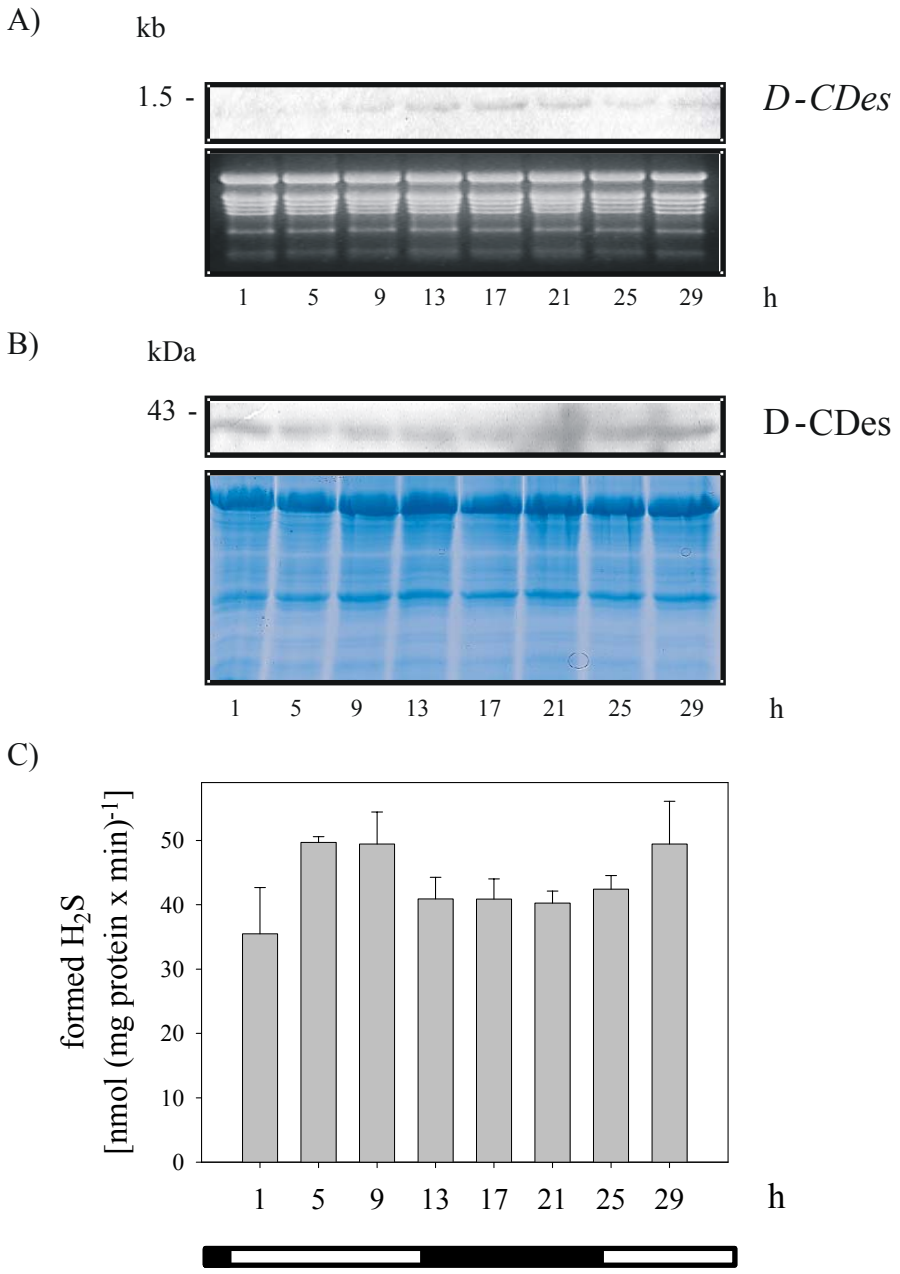


Fig. 6: Expression and activity analyses during a diurnal light / dark cycle. Four-week-old *Arabidopsis* plants were grown in a 12-h light / 12-h dark cycle and the parts above ground were harvested every 4 h and frozen in liquid nitrogen. The analyses were done in the same way as described in Fig. 5. (A) Northern blot, (B) western blot, and (C) determination of specific enzyme activity.

The effects of a 10·different sulfate concentration in the medium were investigated. *Arabidopsis* seeds were germinated in MS medium with 500 µm (high) and 50 µm (low) sulfate concentrations and grown for 18 days. The *Arabidopsis* plants grown at high and low sulfate, respectively, were phenotypically identical. The lower sulfate concentration was chosen because it represents the borderline for normal growth rates. These conditions should reflect the conditions on the field of sulfur-fertilized and nonfertilized *Brassica napus* plants

(E. Schnug, Forschungsanstalt für Landwirtschaft, Braunschweig, Germany, personal communication). After 18 days the shoots were cut and frozen directly in liquid nitrogen. Northern blot analysis indicated an induction of D-CDes expression under low sulfate conditions (Fig. 7A). *yedO* expression was induced by sulfur limitation (Soutourina et al. 2001). The D-CDes protein levels were also increased under the lower sulfate concentration (Fig. 7B). The specific D-CDes activity was not significantly changed by low sulfate (Fig. 7C).

To analyse the effects of cysteine on the expression of D-CDes, *Arabidopsis* suspension cells were treated with 1 mM D- or L-cysteine, respectively, for 2–24 h. No significant differences in either the expression levels or the activity were observed in comparison with the untreated controls (data not shown). In *E. coli* the presence of the *yedO* gene stimulates cell growth in the presence of D-cysteine as the sole sulfur source because the bacterium can utilize H_2S released from D-cysteine. Consequently, *yedO* expression was induced by sulfur limitation (Soutourina et al. 2001).

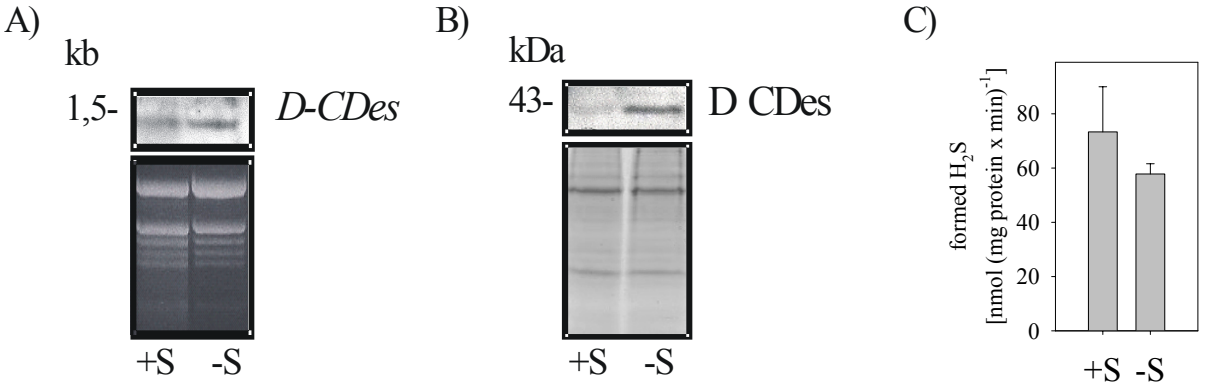


Fig. 7: Expression and activity analyses at high and low sulfate concentration in the growing medium. *Arabidopsis* seeds were germinated in MS with 500 µM (high) and 50 µM (low) sulfate concentration in the medium. The seedlings were grown for 18 days in the same medium. The shoots were cut and frozen directly in liquid nitrogen. The analyses were done in the same way as described in Fig. 5. (A) Northern blot, (B) western blot and (C) determination of specific enzyme activity.

Discussion

Sequence analysis of the D-CDes protein

The PLP-dependent enzymes (B6 enzymes) that act on amino acid substrates are of multiple evolutionary origin. Family profile analysis of amino acid sequences supported by comparison of the available 3D crystal structures indicates that the B6 enzymes known to date belong to four independent evolutionary lineages of paralogous proteins. The α-family includes enzymes that catalyse transformations of amino acids in which the covalency changes are limited to the same carbon atom that carries the amino group forming the imine linkage with the coenzyme. Enzymes of the β-family catalyse mainly β-replacement or β-elimination reactions. The D-alanine aminotransferase and the alanine racemase family are the other two independent lineages (Mehta & Christen 2000). The β-family includes the b-subunit of tryptophan synthase (EC 4.2.1.20), cystathionine β-synthase (EC 4.2.1.22), OAS-TL (EC 4.2.99.8), L- and D-serine dehydratase (EC 4.2.1.13), threonine dehydratase (EC 4.2.1.16), threonine synthases 1 and 2 (EC 4.2.99.2), diaminopropionate ammonia-lyase (EC 4.3.1.15), and the ACC deaminase (Mehta & Christen 2000). The D-CDes protein has to be included in this β-family.

Enzymatic identification and characterization of the YedO homologous **Arabidopsis** *protein as a D-CDes*

The existence of a D-cysteine-specific desulfhydrase in higher plants which converts D-cysteine to pyruvate, H_2S, NH_3 and an unknown fraction was reported for the first time by Schmidt (Schmidt 1982). The ratio of pyruvate and NH_3 was about 1:1, but the inorganic H_2S formation was 2.5-fold higher (Schmidt 1982). It was speculated that 4-methylthiazolidine-1,4-dicarboxylic acid might be formed which was also detected with L-CDes from *Salmonella typhimurium* (Kredich et al. 1973). However, the molecular identity of a plant D-CDes protein could never be elucidated because of instabilities during column protein purification. It was shown that D-cysteine was decomposed by a purified *E. coli* D-CDes stoichiometrically to pyruvate, H_2S and NH_3 (1.43 µmol, 1.35 µmol, and 1.51 µmol, respectively) (Nagasawa et al. 1985). In this work it was demonstrated that an *Arabidopsis* D-CDes protein degraded D-cysteine to pyruvate, H_2S, and NH_3. Interestingly, the PLP-dependent D- elenocystine α,β-lyase from *Clostridium sticklandii* decomposes D-selenocystine into pyruvate, NH_3, and elemental selenium. The enzyme catalyses the β-replacement reaction between D-selenocystine and a thiol to produce S-substituted D-cysteine. Balance studies showed that

1.58 µmol of pyruvate, 1.63 µmol of NH_3, and 1.47 µmol of elemental selenium were produced from 0.75 µmol of D-selenocystine. When the reaction was carried out in sealed tubes in which air was displaced by N_2, 0.66 µmol of H_2Se was produced in addition to elemental selenium. Therefore, the inherent selenium product was labile and spontaneously converted into H_2Se and elemental selenium even under anaerobic conditions. These results and the stoichiometry of the reaction indicated that H_2Se_2 was the initial product (Esaki et al. 1988).

The recombinant D-CDes and the D-CDes protein from *E. coli* have comparable V_{max} values using D-cysteine as substrate (8.6 vs. 13.0 µmol*min^{-1}*mg protein^{-1}) (Nagasawa et al. 1985). The following K_m values using D-cysteine as substrate were determined: spinach, 0.14 mM; YedO, 0.3 mM; D-CDes protein, 0.25 mM. The D-CDes and the YedO protein were inhibited by high D-cysteine concentrations (> 2 mM and > 0.5 mM, respectively). The YedO protein showed some inhibition by L-cysteine with a K_i of 0.53 mM (Nagasawa et al. 1985) whereas the D-CDes protein was inhibited by L-cysteine to a lower extent with a significant reduction (Student's *t*-test at $P < 0.05$) in activity to 53% at 2 mM and to 83% at 0.5 mM.

The addition of dithiothreitol (DTT) to the assay increased the D-CDes activity by about 50%. It was suggested that DTT in the assay might keep D-cysteine in the reduced state (Schmidt 1982). D-CDes from *E. coli* was active as homodimer with 2 mol PLP*mol protein^{-1} (Nagasawa et al. 1985). The D-CDes protein was active as a monomer as demonstrated by size exclusion chromatography (data not shown).

Among different plant species the D-CDes activities are in the same range (Schmidt 1982; Rennenberg et al. 1987; this work). In general, the D-CDes activity was higher in roots than in shoots. In shoots of *Brassica napus* and *Arabidopsis* the specific D-CDes activity was about half as high as the L-CDes activity (data not shown).

The mature protein is localized in mitochondria

Computer programs predicting the intracellular localization of the *Arabidopsis* D-CDes protein predominantly determined mitochondrial localization. The *in silico* results were supported by Western blot analysis of isolated organelles and by the localization studies using fusions with GFP (Figs 3 and 4). In general, the localization predictions of plastidic and mitochondrial proteins are correct for only about 50% of all plant proteins (Bauer et al. 2004). Because of this high degree of uncertainty the prediction results were experimentally proven. All methods applied demonstrated mitochondrial localization for the *Arabidopsis* D-CDes protein.

In experiments done previously the specific D-cysteine activity in *Arabidopsis* was highest in the cytoplasm. In mitochondria the activity was also very high, especially in comparison to L-cysteine desulfhydrase activity (Burandt et al. 2001b). In *Cucurbita pepo* (Cucurbitaceae) plants the D-CDes activity was localized predominantly in the cytoplasm, small amounts of D-CDes activity were shown to be present in the mitochondria; even low D-CDes activity in the chloroplasts was not excluded (Rennenberg et al. 1987). Anderson (1990) demonstrated a nonchloroplastic D-CDes activity. L-CDes activities were found almost exclusively in chloroplasts and mitochondria. It was suggested that the L-CDes activity in the cytoplasmic fraction could be due entirely to broken plastids and mitochondria (Rennenberg et al. 1987). In the same publication H_2S emission from L- and D-cysteine was followed; only the H_2S emission caused by incubation with L-cysteine was inhibited by AOA. The inhibitors acted differently on the L-CDes activities in the different compartments. It was concluded that the degradation of L-cysteine might be catalysed by different types of enzymes (Rennenberg et al. 1987). To solve the contradiction between the two data sets, that published by Rennenberg et al. (1987) and our data, one has to postulate the presence of (an) additional non-PLP cofactor protein(s) with D-CDes activity. Another point to mention is that of species-dependent differences. In both studies species from different plant families have been investigated. In the last years differences between species became more obvious, questioning even the value of the model plant *Arabidopsis*. Chloroplasts are supposed to be the main site for cysteine biosynthesis although OAS-TL proteins are also present in the mitochondria and the cytoplasm (Hell 1997; Leustek 2002). From a physiological point of view the regulation of the cysteine pool by cysteine desulfhydrases in all compartments of the cell would be meaningful.

D-CDes mRNA content, protein level and enzyme activity do not always correlate

In *Arabidopsis* plants D-CDes mRNA levels are regulated by different biotic and abiotic factors, such as light, sulfur nutrition and development, indicating a role in adaptation to changing conditions. The D-CDes protein levels and specific enzyme activities are subject to change but the mRNA, protein and activity levels are not always influenced in the same direction. There are a number of examples where this phenomenon has been observed (e.g. Papenbrock et al. 1999). One could speculate about interaction with other (protein) molecules responsible for mRNA or protein stabilization or enzyme activation or deactivation. Another possibility could be the presence of other proteins with D-CDes activity in *Arabidopsis*, such as protein NP_189241. The study of available microarray data might help to identify

characteristic mRNA expression to focus on a function in the organism. It was shown previously that L-CDes activity in cucurbit plants was stimulated by L- and D-cysteine to the same extent; this process of stimulation itself was light independent. However, a prerequisite produced in the light is necessary to maintain the tissue's potential for stimulation of this enzyme activity (Rennenberg 1983a).

Why do plants have a D-cysteine desulfhydrase?

The function of most D-amino acids in general and especially D-cysteine in almost all living organisms has not been clarified yet. However, in many different plant species a certain percentage of D-amino acids was found. In unprocessed vegetables and fruits about 0.5–3% D-amino acids relative to their L-enantiomers were permanently present (Brückner & Westhauser 2003; Brückner & Westhauser 1994). For technical reasons the relative amount of D-cysteine in comparison to L-cysteine has not been determined so far. Therefore the concentration of D-cysteine in the cell is not exactly known, for L-cysteine a concentration of about 10 µm was determined (Giovanelli et al. 1980). Based on our *in vitro* results we assume that D-cysteine occurs in higher plants, otherwise the D-CDes protein must be specific for other naturally occurring substrates.

A number of functions have been proposed for D-cysteine in plants. The biosynthesis might be specific for L-amino acids, the degradation might occur via the corresponding D-amino acid. This separation could facilitate the regulation of synthesis and degradation by a "compartmentalisation" of amino acid concentration without a special compartment (Schmidt 1982). Incubation of *Arabidopsis* suspension cultures with various non-toxic L- or D-cysteine concentrations (0.1–2 mM) for up to 24 h did not induce either L- or D-CDes activity (data not shown). Probably the desulfhydrase activities constitutively occurring in *Arabidopsis* cells are sufficient to metabolize additional cysteine. Maybe the treatment of intact plants with solutions containing different cysteine concentrations might reveal different results. For a final conclusion the respective concentrations of the enantionmers have to be determined during the feeding experiments. In crude extracts of *E. coli* neither D-CDes nor any activity of an amino acid racemase (to convert L-cysteine to D-cysteine) was detected. Therefore, in the bacterial cell it may be improbable that D-CDes takes part in the regulation of the thiol pools (Nagasawa et al. 1988). Certain biosynthetic routes might use D-amino acids. D-Amino acids could also act as signals for regulatory mechanisms, and then be degraded by specific proteins such as D-amino oxidases (Schmidt 1982). By NMR and MS/MS experiments it was determined that the phytotoxic peptide malformin, produced by *Aspergillus niger*, has the

essential structure of a cyclic pentapeptide containing D-cysteine: cyclo-D-cysteinyl D-cysteinyl L-amino acid D-amino acid L-amino acid (Kim et al. 1993). Malformin caused deformations of plants. One function of D-CDes might be the detoxification of malformin and its components.

How are D-amino acids synthesized?

It was speculated that D-cysteine is not synthesized in higher plants but that it is taken up from the soil where it had been secreted by microorganisms or produced by mycorrhiza (Aldag et al. 1971). It was demonstrated that microbial contamination, or controlled microbial fermentation of edible plants or plant juices, increased amounts and kinds of D-amino acids indicating the ability of microorganisms to produce D-amino acids (Brückner et al. 1995). However, D-CDes activity was demonstrated in suspension cultures of *Arabidopsis* and tobacco growing in Murashige and Skoog minimal medium (MS) minimal medium without the addition of any amino acids (Rennenberg et al. 1987; this work). Therefore, in case D-cysteine is the *in vivo* substrate de novo synthesis has to be assumed and was also established in previous experiments for other D-amino acids as discussed by Brückner & Westhauser (2003). Several enzymes might be synthesizing D-amino acids from L-amino acids such as amino acid oxidases, transaminases, and racemases (epimerases). For example, in pea seedlings the occurrence of D-amino acid aminotransferase was demonstrated (Ogawa et al. 1973). For a number of other amino acids racemases have been identified, e.g. an alanine racemase (Mehta & Christen 2000). It was shown for D-amino acids occurring in animal peptides, such as neuropeptides, that they are formed from L-amino acids by post-translational modifications (Kreil 1997).

Conclusions

This is the first time that a D-CDes from higher plants has been characterized at the molecular level. The analysis of available knockout mutants might help us to understand the function of this enzyme and the occurrence of D-cysteine in general. Interestingly, L-cysteine has a sparing effect on L-methionine when fed to mice, however, D-cysteine does not (Friedman 1991). Therefore D-cysteine-free plants might enhance the nutritional value of plant species short of S-containing amino acids. By producing transgenic D-CDes plants this goal might be reached.

Chapter 8

Isolation and characterization of a second D-cysteine desulfhydrase-like protein from *Arabidopsis*

Introduction

It is well documented that, in general, amino acids are used in the L-form, and enzymes involved in their metabolism are stereospecific for the L-enantiomeres. However, D-amino acids are widely distributed in living organisms (Friedman 1999). Free D-amino acids in the low percentage range of 0.5 to 3% relative to their L-enantiomers are principle constituents of plants (Brückner & Westhauser 2003). The functions of D-amino acids and their metabolism are largely unknown. In several plant species D-cysteine desulfhydrase activity (EC 4.1.99.4) decomposing D-cysteine into pyruvate, H_2S, and NH_3 was measured; however, purification of the protein catalyzing this reaction was not successful due to its instability (Schmidt 1982; Schmidt & Erdle 1983; Rennenberg et al. 1987). Recently, a pyridoxal-5'-phosphate (PLP)-dependent D-cysteine desulfhydrase (D-CDes1) protein was identified in *Arabidopsis thaliana* (L.) Heynh. based on high homology to an *Escherichia coli* protein with D-cysteine desulfhydrase activity (Soutourina et al. 2001; Riemenschneider et al. 2005a). The purified recombinant D-CDes1 protein had a K_m for D-cysteine of 0.25 mM, whereas L-cysteine was not accepted as substrate. Western blot analysis of isolated organelles and localization studies using fusion constructs with the green fluorescent protein (GFP) indicated a subcellular localization of the nuclear encoded D-CDes1 protein in the mitochondria. Expression analysis revealed higher *D-CDes1* mRNA and D-CDes1 protein levels in older plants, during the light phase in a diurnal light/dark rhythm and under sulfate limitation (Riemenschneider et al. 2005a). In the fully sequenced *Arabidopsis* genome (The *Arabidopsis* genome initiative 2000) another sequence showing some degree of similarity with the already characterized *E. coli* and *Arabidopsis* D-cysteine desulfhydrase proteins was found. The initial characterization of this second putative D-cysteine desulfhydrase protein, based on sequence homologies, from *Arabidopsis* (D-CDes2) is described in this paper.

Materials and Methods

To obtain an expression clone the following primer pair was used to amplify a 1,278 bp sequence encoding the full-length D-CDes2 protein from a λYes cDNA library: primer P320 (5'-GGT ACC AAG GTC CAA CGG TCA-3') extended by a *Kpn*I restriction site and primer 321 (5'-GTC GAC ATC TTT CAA GTT AGT G-3') extended by a *Sal*I restriction site. The amplified PCR fragment was ligated into the expression vector pQE-30 (Qiagen, Hilden, Germany). The putative D-CDes2 protein was expressed in *E. coli* according to standard procedures (Riemenschneider et al. 2005a). The purity of the protein preparations was controlled by SDS polyacrylamide gel electrophoresis (PAGE) (Laemmli 1970) and subsequent Coomassie staining. The L- and D-cysteine desulfhydrase activities were measured by the release of H_2S from L- or D-cysteine with the methylene blue method as described (Riemenschneider et al. 2005a). Northern blot analysis was done as described (Riemenschneider et al. 2005a).

Results

An open reading frame encoding a protein with D-Cysteine desulfhydrase activity called D-CDes1 (At1g48420) had been characterized in *Arabidopsis* (Riemenschneider et al. 2005a). By *in silico* analysis a second protein (At3g26115, D-CDes2) containing a PLP-binding site with 19.1% identity and 34.5% similarity to the D-CDes1 protein could be identified. The complete coding region of 1,284 bp was obtained by PCR. The deduced D-CDes2 protein consists of 427 amino acids including the initiator methionine and excluding the stop terminating amino acid. The protein has a predicted molecular mass of 47.4 kDa and a pI of 8.5. It contains high amounts of the sulfur amino acids cysteine (7 residues) and methionine (16 residues). According to several programs predicting the intracellular localization of proteins in the cell (http://www.expasy.ch/tools), the protein might possess an N-terminal extension indicating the import into mitochondria (TARGETP), whereas PSORT predicted a localization either in peroxisomes (0.37) or in mitochondria (0.36). In PSORT a protease cleavage site between amino acid 45 and 46 counting from the start methionine was predicted, indicating a pre-sequence of 45 amino acids. The mature protein would have a molecular mass of 42.3 kDa and a pI of 7.9. Preliminary data obtained by the analysis of transiently transformed *Arabidopsis* protoplasts with fusion constructs with GFP indicates a localization in mitochondria (data not shown).

The recombinant *Arabidopsis* D-CDes2 protein including the targeting peptide was expressed in *E. coli* cells and 3 h after induction the protein accumulated up to 5% of the total *E. coli* protein in the cells. The protein could be purified near to homogeneity by affinity chromatography (Fig. 1). For the D-cysteine desulfhydrase activity a V_{max} of 37.8±6.9 nmol (min mg protein)$^{-1}$ was determined. The protein accepted also L-cysteine as substrate and showed even a higher specific L-cysteine desulfhydrase activity of 88.5±19.2 nmol (min mg protein)$^{-1}$. The addition of pyridoxine and thiamin to the dialyzing buffer or to the enzyme assay solution did not increase enzyme activity of this PLP-dependent protein.

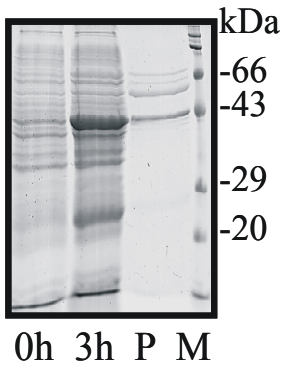


Fig. 1: SDS-PAGE analysis of *E. coli* carrying *Arabidopsis* cDNA encoding the D-CDes2 protein cloned into the pQE-30 expression vector. Samples were denatured in the presence of 56 mM DTT and 2% SDS, heated for 15 min at 95°C, and centrifuged. Aliquots of the supernatant were separated by SDS-PAGE. Lanes described from the left to the right: 0 h, protein extract of transformed *E. coli* strain XL1-blue shortly before induction of the culture with isopropyl-β-D-1-thiogalactopyranoside (IPTG); 3 h, transformed *E. coli* strain XL1-blue protein extract 3 h after induction with IPTG; P, protein purified by Ni^{2+}-affinity chromatography (10 µg); M, protein marker (Roth). The molecular masses of the marker proteins are given in kDa on the right.

Expression analysis was done at different developmental stages and after pathogen attack. *Arabidopsis* plants were grown for 10 to 45 days and the parts above ground were harvested every week. The *D-CDes2* mRNA levels were highest in the youngest plants and decreased in older plants (Fig. 2A). The inoculation of leaves with *Erwinia carotovora* ssp. *carotovora* or *Pseudomonas syringae* pv *maculicola* did not influence mRNA expression levels in comparison to controls (Fig. 2B).

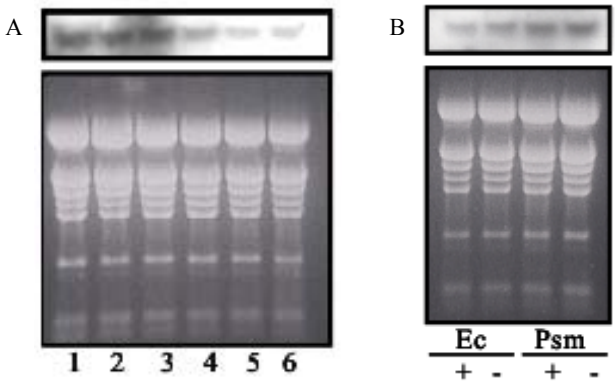


Fig. 2: Northern blot analysis. **A** *Arabidopsis* plants were grown in the greenhouse for one to six weeks and all plant tissue above ground was used for the analysis. **B** Leaves of *Arabidopsis* plants were inoculated with *Erwinia carotovora* ssp. *carotovora* (Ec) and *Pseudomonas syringae* pv *maculicola* (Psm). The plant parts above ground were harvested 6 days after inoculation and used for analysis. As control leaves inoculated with suspension medium were used. Total RNA was extracted, 20 µg RNA was loaded in each lane and blotted. To prove equal loading of the extracted RNA the ethidium bromide-stained gel is shown at the bottom. *D-CDes2* cDNA was labeled with digoxigenin by PCR.

Discussion

On the basis of sequence similarities to *Arabidopsis* D-cysteine desulfhydrase1 a second protein was identified in this model plant which was assumed to act as a D-cysteine desulfhydrase *in vitro* and *in vivo*. The coding sequence was amplified and heterologously expressed in *E. coli*. Interestingly, the purified protein catalysed the release of H_2S from D-cysteine and also from L-cysteine. To our knowledge this is the first cysteine desulfhydrase enzyme which catalyses the degradation of L- and D-cysteine. All other L-cysteine desulfhydrases investigated so far accept only L-cysteine (e.g. Jaschkowitz & Seidler 2000; Tchong et al. 2005). However, it can not be excluded that also other substrates except cysteine might be the *in vitro* and *in vivo* substrates. In comparison to D-CDes1 without pre-sequence the specific D-cysteine desulfhydrase activity was much lower [2.5 µmol (min mg protein)$^{-1}$ in comparison to 38 nmol (min mg protein)$^{-1}$]. Possibly the protein might have to be expressed without the pre-sequence in its mature form to obtain higher enzyme activities as was also the case for D-CDes1.

Computer programs predicting the intracellular localization of this *Arabidopsis* D-CDes2 protein predominantly determined mitochondrial or peroxisomal localization. Preliminary experimental localization studies using GFP fusions constructs transiently transformed into *Arabidopsis* protoplasts indicated a localization in mitochondria. Probably both proteins with D-cysteine desulfhydrase activity are imported into the same compartment.

The expression of D-CDes2 was higher in younger than in older *Arabidopsis* plants whereas the expression of D-CDes1 was almost constant during aging (Riemenschneider et al. 2005a). Therefore both genes might have specific roles in the organism. The *D-CDes* genes might be expressed in different tissues which should be analysed in the future. Both bacterial pathogens used for inoculation did not influence the *D-CDes2* mRNA levels, at least in the long term experiment lasting several days. Pathogens might induce cysteine desulfhydrases in the plant and the release of H_2S might be one strategy of the plant's defence system (Bloem et al. 2004).

Our major goal for the future is to clarify the respective role(s) of D-cysteine and D-/L-cysteine desulfhydrases in the plant organism. A number of functions were proposed for D-cysteine: The biosynthesis might be specific for L-amino acids, the degradation might occur via the corresponding D-amino acid. This separation could facilitate the regulation of synthesis and degradation by a compartmentation of amino acid concentration without a special compartment (Schmidt 1982). For a final conclusion the respective concentrations of the enantiomeres have to be determined in the plant organism and during the feeding experiments. Certain biosynthetic routes might use D-amino acids. D-amino acids could also act as signals for regulatory mechanisms, and then be degraded by specific proteins such as D-amino oxidases (Schmidt 1982). It was determined that the phytotoxic peptide malformin, produced by *Aspergillus niger*, has the essential structure of a cyclic pentapeptide containing D-cysteine: cyclo-D-cysteinyl D-cysteinyl L-amino acid D-amino acid L-amino acid (Kim et al. 1993). Malformin caused deformations of plants. One function of D-cysteine desulfhydrase might be the detoxification of malformin and its components. L-cysteine desulfhydrases might be involved in direct defence against pathogen attack by releasing H_2S as previous results indicate.

Chapter 9

General discussion

The functions of *O*-acetyl-L-serine(thiol)lyase isoforms and cysteine desulfhydrases

Sulfate is the primary source of sulfur for plants. Sulfate is taken up by the roots from the rhizosphere, reduced via several enzymatic steps, and finally *O*-acetyl-L-serine(thiol)lyase (OAS-TL) catalyses the biosynthesis of cysteine from H_2S and *O*-acetyl-L-serine (OAS) (for a review see De Kok et al. 2002b and Leustek 2002). In its free form OAS-TL isoforms are also able to catalyse in a side reaction the degradation of cysteine by releasing H_2S.

Experiments with non-sequenced transgenic potato plants have been done to clarify the particular role of the different OAS-TL isoforms in cysteine synthesis and homeostasis in plants (Chapter 2). The role of OAS-TL isoforms in H_2S release in a side reaction was another point of interest. Interestingly, the plants carrying an antisense construct against the cytosolic *OAS-TL* showed less enzyme activity to form cysteine, but also the capacity to release H_2S decreased drastically. One explanation could be that the cytosolic OAS-TL isoform is involved in H_2S release because in *Arabidopsis* it was possible to localise putative proteins with cysteine desulfhydrase activity only in mitochondria, in plastids and the nucleus (Kushnir et al. 2001; Leon et al. 2002; Pilon-Smits et al. 2002; Riemenschneider & Papenbrock, unpublished results). The reduced capability of transgenic potato plants to produce cysteine indicated by the accumulation of OAS and the precursor serine led to the speculation that the reduction in OAS-TL protein resulted in fewer binding partners for L-serine acetyltransferase (SAT), which causes the increase in serine levels. If SAT is bound to OAS-TL in the multi-enzyme complex, called cysteine synthase (Kredich et al. 1969; Wirtz et al. 2001), OAS can be synthesized. However it can not completely used for cysteine formation because of the reduced amounts of free OAS-TL protein (Hell et al. 2002). This led to the balance of cysteine homeostasis. The ability of OAS-TL to release H_2S in a side reaction is with the binding of OAS-TL in the multi-enzyme complex inactivated (Burandt et al. 2002).

One part of the short-term H_2S exposure experiment was to determine the influence of H_2S on gene and protein levels of the OAS-TL isoforms (Chapter 3). In the case of the fumigation experiment free OAS-TL is active to reveal the detoxification of H_2S. One could postulate

that after H_2S fumigation only the mitochondrial OAS-TL isoform was induced because even low H_2S concentrations act toxically on the respiratory rate (Beauchamp et al. 1984). The total OAS-TL enzyme activity was not significantly changed after H_2S fumigation. However, it was shown that transgenic plastidial OAS-TL sense tobacco plants possessing two to three times higher OAS-TL activities were resistant to 100 µl l^{-1} H_2S applied for 2 and 24 h. Only after 6 d of this extremely high H_2S concentration also the sense plants showed necrotic lesions comparable to the lesions on wild-type plants already visible after 2 h of H_2S fumigation (Youssefian et al. 1993). The finding that the catalytic capacity of OAS-TL exceeds sulfur assimilation needs by several 100-fold (Schmidt & Jäger 1992) may indicate a direct role of the OAS-TL enzyme in the use and/or the detoxification of atmospheric H_2S without enhancing the overall enzyme activity or the *OAS-TL* mRNA expression. The tendency of decreasing OAS concentrations after exposure of *Arabidopsis* plants to H_2S. But also the slight changes in the cysteine desulfhydrase enzyme activity could support this explanation. Cysteine desulfhydrase might also catalyse the synthesis of cysteine as was shown for *Spinacia oleracea* extracts (Poortinga et al. 1997).

Cysteine desulfhydrases might also play a role in the detoxification of cysteine by releasing H_2S and hold therefore the cysteine content in plants at very low levels (Giovanelli et al. 1980). CDes catalyse the degradation of cysteine, resulting in the formation of H_2S, pyruvate and ammonium, or in elemental sulfur and alanine (Mihara & Esaki 2002; Rennenberg et al. 1987; Zheng et al. 1993). Experiments done with *B. napus* under sulfur starvation have shown that the cysteine desulfhydrase enzyme activity decrease in plants with sulfur deficiency but the expression of the respective mRNAs has not been increased (Chapter 6). The cysteine desulfhydrase activity seems not to be influenced and ensures therefore that the necessary sulfur-containing products, glutathione (GSH) and other related compounds are not degraded. The GSH and cysteine contents decreased in sulfur-deficient plants about 50%. Because of enough nitrogen supply, sulfur is the limiting factor. Blake-Kalff et al. (1998) could observe that plants with higher nitrogen supply grew faster and therefore had an increased demand for sulfur. This was reflected by a faster decrease in the concentration of insoluble sulfur. Generally, at low nitrogen and minus sulfur supply the internal concentration of sulfur and nitrogen is more balanced. This balance between nitrogen and sulfur is closely related and has been reported previously (Hesse et al. 2004). Dependent on the sulfur status of the investigated plants it would be interesting to measure the OAS-TL enzyme activity and the influence of pathogens. Maybe one could obtain more information about the H_2S release from OAS-TL in a side reaction to clarify the importance of OAS-TLs. Experiments done with

different oilseed rape lines have namely shown that the higher the sulfur content in the plants was, the lower the activity of the H_2S-releasing enzyme L-cysteine desulfhydrase (L-CDes) and the higher the OAS-TL activity (Burandt et al. 2001a).

A further component for H_2S release was the identification of an *Arabidopsis* gene encoding a putative D-cysteine desulfhydrase (D-CDes) based on a high homology to an *E. coli* protein called YedO (Soutourina et al. 2001), which made it possible to link the long known plant D-cysteine desulfhydrase activity (Schmidt 1982; Schmidt & Erdle 1983) with a specific gene and protein (Chapter 7). Experiments done in the past could clearly separate the D-cysteine desulfhydrase from the L-cysteine desulfhydrase activity on a DEAE-sephacel column (Schmidt & Erdle 1983) but the related gene could not be isolated.

The D-CDesI protein localised in mitochondria uses D-cysteine as substrate for the degradation to pyruvate, ammonium and H_2S. The observed enzyme activity underlies a diurnal rhythm and increases with increasing plant age. Burandt et al. (2001c) observed also a dependence of L-cysteine desulfhydrase activity to aging and the diurnal rhythm. Maybe protein degradation plays a role in the increase of enzyme activity during the process of aging. But it could also be a way to avoid the accumulation of toxic levels of cysteine in the cell described above.

In general, D-amino acids were detected in various plants (Friedman 1999). Free D-amino acids in the low percentage range of 0,5 to 3% relative to their L-enantiomers are principal constituents of plants (Brückner & Westerhauser 2003). The functions of D-amino acids and their metabolism are largely unknown. Only a few other PLP-dependent enzymes acting on D-amino acids have been found (Dowhan & Snell 1970; Nagasawa et al. 1982; 1985; 1988; Schmidt 1982). A second D-CDes sequence in *Arabidopsis* with 34,5% similarity on the amino acid level to D-CDesI was identified (Chapter 8). Because of different expression patterns, e.g. during aging, both D-CDes proteins might have specific roles in the organism. The *D-CDes* genes might be expressed in different tissues and should be analysed in further experiments.

The transformation of L-amino acids in D-amino acids might constitute a form of compartmentation of amino acid concentration without a special compartment (Schmidt 1982), suggesting that D-CDes might really have a detoxification function. This means that only L-CDes might be involved in direct defence against pathogen attack.

Is there a correlation between H_2S and SIR?

The enhanced emission controls already mentioned led to a dramatic reduction in the atmospheric deposition of sulfur in recent years (Dämmgen et al. 1998). Renunciation of sulfur-containing fertilizers and the higher sulfur demand of high-yielding crops have led to widespread macroscopic sulfur deficiency. Increasing deficiency symptoms (Schnug et al. 1995) and an increase of diseases led to the opinion that there might be a relationship between the sulfur supply and defence mechanisms against diseases. The hypothesis of sulfur-induced resistance (SIR) was defined. The physiological background for the natural resistance of crops against certain pests and diseases is thought to be related to increasing H_2S emission of the plants, which is highly toxic and thus may act as a fungicide (Beauchamp et al. 1984; Sekiya et al. 1982a).

H_2S may be released before or after cysteine formation (Giovanelli 1990). Two candidates which could be responsible for H_2S release are CDes and OAS-TL isoforms. The fact that the OAS-TL and L-cysteine desulfhydrase enzyme activity were higher in sulfur deficient plants (Salac 2005; Chapter 5) indicated that under conditions of sulfur deficiency the sulfur metabolism is activated and the participating enzymes are up-regulated. There are two possible explanations: sulfur deficient plants were more susceptible to fungal diseases and therefore they increased the metabolic pathways, which are involved in plant protection. The second explanation could be that sulfur deficient plants already had a stronger fungal infection and the mechanisms of SIR were activated. Therefore, the release of H_2S can be a mechanism of protection to prevent a fungal attack, or the answer to a fungal attack, or perhaps both mechanisms work at the same time. Interestingly, in line Lion and genotype 15157 from *B. napus* the enzyme activity of L-cysteine desulfhydrases and D-cysteine desulfhydrases have shown any changes neither in plants with nor without sulfur supply (Chapter 6). That might implicate that pathogen attack seems to play an important role in the behaviour of the participating enzymes. Experiments done with *B. napus* and the pathogens *L. maculans*, *B. cinerea* and *P. brassicae* have also shown a clear coherence from plants under sulfur deficiency and enhanced disease susceptibility (Dubuis et al. 2005). The authors have two arguments why sulfur deficient plants become more susceptible to pathogens. First, the increased susceptibility is caused by the specific effect of sulfur deficiency on the accumulation of sulfur containing defence compounds such as phytoalexins, glucosinolates, and cysteine-rich antifungal polypeptides which can play important roles in disease resistance. Second, sulfur deficiency leads to a general reduction of fitness and global weakening of the plants that causes generally enhanced susceptibility to stress.

Interestingly, both enzymes, OAS-TL and L-CDes showed a positive correlation in infected as well as in uninfected leaf discs (Chapter 5). Burandt et al. (2001a) found an inverse relationship of both enzyme activities for different genotypes of oilseed rape. These crops received the same rate of sulfur fertilization but differed in their susceptibility to different fungal diseases. These different oilseed rape genotypes act differently on bacterial attack. Line 1 with the highest total sulfur content (Burandt et al. 2001a) has shown the lowest L-cysteine desulfhydrase enzyme activity and the highest OAS-TL activity after inoculation with three different bacteria. After fungal infection the positive relationship between OAS-TL and L-CDes indicates that the activity of OAS-TL is also increasing, but not as a direct result of the infection, but as a reaction to the activity of L-cysteine desulfhydrase which is consuming cysteine, the product of the OAS-TL reaction. On the other hand in *OAS-TL* antisense potato plants the cytosolic OAS-TL isoforms seems to be directly involved in H_2S release (Chapter 2). The L-cysteine desulfhydrase activity increased due to infection with *P. brassicae*. The fact that OAS-TL was not significantly up-regulated while the product of the reaction, cysteine, increased strongly, probably shows that the enzyme activity was high enough to allow a fast turnover from sulfate to cysteine. These results suggest that OAS-TL is not actively increasing the H_2S release with infection, but can only participate in an increasing H_2S release in a passive way.

The influence of the fungal infection on the cysteine and GSH contents and L-cysteine desulfhydrase activity was even stronger than that of the sulfur nutritional status of the crop, which also had a significant positive effect on the different sulfur fractions. These results might support the concept of SIR. With a better sulfur supply more cysteine and GSH are metabolized and, therefore, the potential of SIR is increasing. However, in the paper of Blake-Kalff et al. (1998) the authors concluded that in *B. napus* neither GSH nor glucosinolates were major sources of sulfur during sulfur deficiency.

Elemental sulfur is the only known inorganic phytoalexin (for more information see Williams & Cooper 2004) and is regarded as the man's oldest fungicide. Impregnation of vascular tissues with elemental sulfur could be a direct relevance to resist against vascular fungi by proving an effective barrier (Cooper 2000; cited in Williams et al. 2002). Williams et al. (2002) observed in resistant tomato plants 6 to 10 µg S^0 g^{-1} excised from vascular tissue. These levels were greater than required for inhibition of *V. dahliae* spore germination. CDes are discussed to be involved to the formation of elemental sulfur.

The minimum of H_2S release necessary to yield a fungicidal effect is calculated to 10 µM H_2S h^{-1} (cited in Bloem et al. 2004; Haneklaus et al. 2006). Fumigation experiments

with *Arabidopsis* have shown that the H_2S emission from plants acting against pathogens can only be in the range up to 0,5 µl l^{-1}. Higher H_2S exposure led to oxidative stress symptoms (Chapter 3). H_2S is very reactive and complexes with high affinity to metallo-groups in proteins and cause with it the primary biochemical basis for the phytotoxicity of H_2S (Beauchamp et al. 1984; De Kok et al. 1998; 2002a).

To clarify the question of the title; yes, there seems to be a correlation between H_2S-releasing enzymes and reactions on pathogen attacks. However, newest discussions lead to the opinion that sulfur-induced resistance is a misnomer because sulfur nutrition does not induce resistance but rather enhance defence operations and might even act indirectly by improving general plant performance under biotic stress (Rausch & Wachter 2005). The term SIR should be replaced by the term sulfur-enhanced defence (SED).

In which range CDes proteins play a role in plant protection cannot be clarified in this work, but there is a correlation of desulfhydrases to sulfur nutrition. In sulfur starvation experiments with *B. napus* plants there was a higher *CDes* mRNA accumulation observed in stems independent from the sulfur status of the plants (Chapter 6). Taking into account that Williams & Cooper (2003; 2004) and Williams et al. (2002) could find an accumulation of elemental sulfur in vascular tissue might indicate participation of CDes. But this would also mean that if the precursor for the degradation to H_2S is rather limited, sulfur supply will only enhance the possibility of releasing H_2S. High *CDes* mRNA accumulation and cysteine desulfhydrase activity could be detected (Chapter 6) but under sulfur starvation compounds, e.g. glutathione, would not be adequate for a fast and efficient answer to pathogen attack.

What is the best method to measure H_2S?

Next to true CDes also OAS-TL isoforms might be involved in the release of H_2S, in a side reaction (Burandt et al. 2002; Schmidt 1977a, b). Two established enzyme assays generally used to determine the enzyme activity of OAS-TL and CDes are known (Siegel 1965; Gaitonde 1967). Results discussed above have shown that it is useful to measure both the synthesis and the degrading enzyme activity. But in the final analysis only one assay was used to determine the capacity of H_2S release *in vitro*.

We are interested in the amounts of H_2S released *in vivo*. How much H_2S is released from a plant under different conditions and would it be enough to stop pathogen attack? For years different methods have been tested. Volatile sulfur compounds have been collected with floated plastic or nylon bags (McEwan & MacFarlane Smith 1998). Plants or plant parts have

been placed in glass containers or dynamic gas exchange cuvettes (Rennenberg et al. 1990). In the end the collected samples had to be determined by gas chromatography.

Contacts to a marine biology scientist made it possible for us to test a H_2S microsensor (Chapter 4). In general this equipment is used for the detection of H_2S in bacteria and liquids (Schulz & Jørgensen 2001; Schulz & de Beer 2002). This microsensor is sensitive enough to measure H_2S concentration within seconds. The tip size is small enough to measure different points on and in an *Arabidopsis* leaf. But there are still some problems with the use of this equipment because the method to measure H_2S concentrations from plants is still in its "children's shoes". One of the problems is the reproduction. Problems with the damage of the leaf tissue and exact measurements in different leaf tissues are placed in the second order. A typically leaf of *Arabidopsis* is dependent from the age 200 to 400 µm thick. In the different cell layers the relations of apoplast to symplast are different. The pH differs in the different cell compartments (Burandt et al. 2002). Because of the different solving behaviour of H_2S in different pH values the exact actual H_2S concentration cannot be determined without pH measurements at the same place.

The development of a method combining established methods for H_2S detection *in vitro* with method applied for ion quantification with microsensors could be helpful and should be one part planned for the future. Therefore, a microsensor has to be constructed which makes possible to measure the pH and the H_2S concentration at the same time. The tip size has to be thinner to focus in a respiration cave or in one cell. Then is might be also possible to measure in one cell without damaging the membrane potential. Measurements *in vivo* would be possible. Otherwise combinations of different long-known methods have to be used to calculate the H_2S release from living plants.

Conclusion

It was possible to correlate the increasing cysteine desulfhydrase enzyme activity with the pathogen infection of plants under sulfur starvation. In the case of sulfur deficiency and higher atmospheric H_2S supply plants have not shown any significant differences in their enzyme activities. Interestingly, in transgenic potato plants the H_2S-releasing side seemed to be dominated by OAS-TL enzyme activity. The expression studies of CDes in different plant organs have shown highest mRNA accumulation in stems. The use of the *in situ* hybridisation and the development of a plant-useable H_2S microsensor are experiments to work for in the future to evaluate the influence of CDes in plant protection and to detect H_2S concentration *in vivo*.

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Erklärung

Ich versichere, dass die Dissertation selbständig verfasst und die benutzten Hilfsmittel und Quellen, sowie gegebenenfalls die zu Hilfsleistungen herangezogenen Institutionen, vollständig angegeben wurden und die Dissertation nicht schon als Diplomarbeit oder ähnliche Prüfungsarbeit verwendet worden ist.

Hannover, den 19. Januar 2006 Anja Riemenschneider

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Bloem E, Riemenschneider A, Volker J, Papenbrock J, Schmidt A, Salac I, Haneklaus S, Schnug E. 2004. Sulphur supply and infection with *Pyrenopeziza brassicae* influence L-cysteine desulphydrase activity in *Brassica napus* L. J Exp Bot 55: 2305-2312.

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