FUNCTIONAL CHARACTERISATION OF SULFURTRANSFERASE PROTEINS IN HIGHER PLANTS

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SUMMARY

Sulfurtransferases (Str) catalyse the transfer of a sulfur atom from suitable sulfur donors to nucleophilic sulfur acceptors. Str proteins are present in organisms of all three phyla but although several functions were proposed, their biological role is yet to be determined. The research aims at the elucidation of the properties and functional relevance *in vivo* of the 20 members of the heterogeneous Str multi protein family in *Arabidopsis thaliana*. The 20 putative Str proteins, each containing at least one rhodanese (Rhd) domain, have been classified into six groups according to sequence homologies. In this work studies on structure, localisation and expression as well as *in vitro* and *in vivo* activity of several of the proteins and characterisation of transgenic plants have been conducted.

By fluorescence spectroscopic analyses the prolonged linker sequence in the two-domain protein AtStr1 was shown to be important in maintaining the correct conformation and stability of the protein and thereby also in enzyme activity. In AtStr1C332S the catalytic cysteine residue has been substituted for serine. It could be shown that apart from the loss of the persulfuration site also conformational changes of the whole protein structure led to the loss of activity. Further indications are provided for a direct involvement of C339 in the sulfur transfer catalysis.

To enzymatically characterise the AtStr of group IV *in vitro,* the respective proteins have been overexpressed in *Escherichia coli*. Since the proteins were expressed in an insoluble state they could not be characterised on the catalytic level, yet. Thus, substrate specificity and kinetic properties of the proteins remain to be elucidated.

Additionally their intracellular location has been addressed. Results from transient expression of fusion constructs with the green fluorescent protein (GFP) in *Arabidopsis* protoplasts indicate a localisation of AtStr9 in chloroplasts and of AtStr10 in mitochondria while AtStr11 may display a dual-localisation in chloroplasts and peroxisomes.

In expression studies under various environmental conditions the expression of the members of groups I, IV and VI of the *Str* gene family in *Arabidopsis* was shown to be differentially affected by distinct environmental factors, such as light/dark rhythm, developmental stage and nutritional status, indicating that the various members may serve distinct functions within the plant.

To elucidate the biological function of AtStr *in vivo*, *Arabidopsis* T-DNA knockout mutants of *AtStr1*, *AtStr2*, *AtStr11* and *AtStr15* as well as transgenic *Nicotiana tabacum* plants heterologously overexpressing *AtStr1* from *Arabidopsis* were initially characterised. A proposed function of AtStr15 in molybdenum cofactor biosynthesis could be excluded, while AtStr1 might be involved in plant defence reactions following pathogen attack. The interruption of *AtStr2* and *AtStr11* produced strong phenotypes of misshaped first leaves and stunted growth, respectively. The proteins might act in regulating certain pathways by signalling or activation and deactivation of other proteins.

The involvement of Str activities in biotic and abiotic stress response reactions of plants appears quite likely. However, the proposed functions can only be speculative since mutants still have to be properly verified. The results obtained in this work are an essential pre-requisite and an excellent basis for further investigations on the biological functions of Str in plants.

Keywords: *Arabidopsis thaliana*; functional genomics; GFP; subcellular localisation; sulfurtransferases

ZUSAMMENFASSUNG

Sulfurtransferasen (Str) katalysieren den Transfer eines Schwefelatoms von einem geeigneten Schwefeldonator auf einen nucleophilen Akzeptor. Str sind in Organismen aller drei Domänen vorhanden. Doch obwohl verschiedene Funktionen der Proteine vorgeschlagen wurden, ist ihre biologische Rolle noch immer nicht bekannt. Ziel der Forschung ist die Ermittlung der Eigenschaften und funktionellen Bedeutung der 20 Mitglieder der heterogenen Str-Multiproteinfamilie in *Arabidopsis thaliana.* Die 20 mutmaßlichen Str Proteine weisen jeweils mindestens eine Rhodanese (Rhd) Domäne auf und wurden anhand von Sequenzhomologien in sechs Gruppen unterteilt. Inhalt der vorliegenden Arbeit sind Untersuchungen der Struktur, der Lokalisation und Expression, wie auch der Aktivität mehrerer Str Proteine *in vitro* und *in vivo* sowie die Charakterisierung transgener Pflanzen.

Durch fluoreszenz-spektroskopische Analyse konnte gezeigt werden, daß die verlängerte Verbindungssequenz der zwei Domänen in AtStr1 eine wichtige Rolle in der Aufrechterhaltung der korrekten Konformation und dadurch auch für die Enzymaktivität spielt. In AtStr1C332S wurde der katalytisch aktive Cysteinrest durch Serin ersetzt. Neben dem Verlust der Persulfurierungsstelle sind auch konformative Veränderungen der gesamten Proteinstruktur für den Verlust der Aktivität verantwortlich. Zudem konnten weitere Hinweise für eine direkte Beteiligung von C339 am Schwefeltransfer erbracht werden.

Um die AtStr der Gruppe IV *in vitro* enzymatisch zu charakterisieren, wurden die entsprechenden Proteine in *Escherichia coli* überexprimiert. Da die Proteine in unlöslicher Form exprimiert wurden, war die Charakterisierung auf katalytischer Ebene noch nicht möglich. Die Frage nach Substratspezifität und den kinetischen Eigenschaften der Proteine muß daher zunächst unbeantwortet bleiben.

Ergänzend wurde die intrazelluläre Lokalisation der Proteine behandelt. Ergebnisse transienter Expression von Fusionskonstrukten mit dem grün fluoreszierenden Protein (GFP) in *Arabidopsis* Protoplasten deuten auf eine chloroplastidäre Lokalisation von AtStr9 sowie eine mitochondriale Lokalisation von AtStr10 hin, während AtStr11 möglicherweise eine duale Lokalisation in Chloroplasten und Peroxisomen aufweist.

Expressionsuntersuchungen von Mitgliedern der Gruppen I, IV und VI der *Str*-Genfamilie in *Arabidopsis* unter verschiedenen äußeren Bedingungen zeigen, dass die Expression der Gene in unterschiedlicher Weise durch so verschiedene äußere Faktoren wie Licht/Dunkel-Rhythmus und Entwicklungs- und Ernährungsstatus bestimmt sind. Dies lässt auf unterschiedliche Funktionen der verschiedenen Mitglieder innerhalb der Pflanze schließen.

Um die biologische Funktion von AtStr *in vivo* zu klären, wurde die Charakterisierung von *Arabidopsis* T-DNA Insertionsmutanten von *AtStr1, AtStr2, AtStr11* und *AtStr15* sowie von transgenen *Nicotiana*-Pflanzen, die *AtStr1* aus *Arabidopsis* heterolog überexprimieren, begonnen. Eine vorgeschlagene Funktion von AtStr15 in der Biosynthese des Molybdän-

Cofaktors konnte ausgeschlossen werden, hingegen könnte AtStr1 in pflanzlichen Abwehrreaktionen in Folge eines Pathogenangriffs eine Rolle spielen. Die Ausschaltung von *AtStr2* und *AtStr11* führte zur Ausprägung deutlicher Phänotypen in Form von mißgestalteten ersten Blättern bzw. gestauchtem Wuchs. Die Proteine agieren möglicherweise in der Regulierung bestimmter stoffwechselphysiologischer Abläufe durch Signalwirkung oder Aktivierung und Deaktivierung von anderen Proteinen.

Das Mitwirken von Str-Aktivitäten in der Reaktion der Pflanze auf biotischen und abiotischen Stress scheint sehr wahrscheinlich. Die vorgeschlagenen Funktionen können jedoch lediglich spekulativ sein, da die Mutanten zunächst abschließend verifiziert werden müssen. Die Ergebnisse dieser Arbeit stellen eine notwendige Vorraussetzung dar und bilden darüber hinaus eine exzellente Grundlage für weiterführende Untersuchungen der biologischen Funktionen von Str in Pflanzen.

Schlagwörter: *Arabidopsis thaliana*; Funktion; GFP; intrazelluläre Lokalisation; Sulfurtransferasen

ABBREVIATIONS

ΔF	fluorescence intensity
2YT	2x yeast tryptone (medium)
3-MP	3-mercaptopyruvate
aa	amino acid(s)
ass.	associated
A. thaliana	*Arabidopsis thaliana*
AtStr	*Arabidopsis thaliana* sulfurtransferase(s)
A.U.	fluorescence arbitrary units
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
bis-ANS	1,1'-bis(4-anilino)naphthalene-5,5'disulfonic acid
BP	band pass
c, CP	chloroplast
CD	circular dichroism
CE	capillary electrophoresis
cfu	colony forming unit(s)
Cys	cysteine
db	database
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
dpi	days past inoculation
dpg	days past germination
DTT	dithiothreitol
E	sulfur-free enzyme
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylenegycoltetraacetic acid
er, ER	endoplasmic reticulum
ES	sulfur-substituted enzyme
EST	expressed sequence tags
F_0	original fluorescence intensity
F_{obs}	observed fluorescence intensity
FW	fresh weight
exp	experimental
GFP	green fluorescent protein
His	histidine
ID	identification
IPTG	isopropyl-β-D-galactoside
kDa	kilo Dalton
LB	Luria Bertani (medium)
LP	long pass
M	protein resp. DNA standard
m, MT	mitochondrial, mitochondrium
MAP	mitogen-activated protein
MoCo	molybdenum cofactor
mRNA	messenger ribonucleic acid
MRW	mean residue weight
MS	Murashige & Skoog (medium)

MW	molecular mass
NBT	nitroblue tetrazolium
no.	number
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDA	photodiode array
PER	peroxisome
pI	isoelectric point
pred	predicted
Rhd	rhodanese(s)
Rhd-S	sulfur-substituted rhodanese
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
rpm	rounds per minute
SAG	senescence associated gene
SDS	sodium dodecyl sulfate
sp.	species
SP	signal peptide
Str	sulfurtransferase(s)
TB	terrific broth (medium)
T-DNA	transfer deoxyribonucleic acid
tm	transmembrane
TP	targeting peptide
TS	thiosulfate
UV	ultra violet
WT, wt	wild-type

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CONTENTS

CHAPTER I **1**
GENERAL INTRODUCTION 1
General and structural aspects of sulfurtransferase proteins 1
Subcellular localisation of sulfurtransferase proteins 4
Putative *in vivo* functions of sulfurtransferase proteins 5
Aims and experimental program of this thesis 6
CHAPTER II **8**
CONFORMATIONAL STUDIES ON *ARABIDOPSIS* SULFURTRANSFERASE AtStr1 ANALYSED BY SPECTROSCOPIC METHODS 8
Abstract 8
Introduction 8
Material & Methods 10
Expression and purification of the AtStr proteins in *E. coli* 10
Investigations of the proteins by fluorescence spectroscopy 10
Structural analysis of proteins by circular dichroism 11
Limited proteolysis 11
Western blot analysis 11
Miscellaneous 11
Results 12
Status of persulfuration and the influence of substrates 12
Role of the linker: results of fluorescence spectroscopy 14
Role of the linker: results obtained by circular dichroism spectroscopy analysis in the near UV range 14
Role of the linker: results obtained by circular dichroism spectroscopy in the far UV range 16
Partial tryptic proteolysis 17
Discussion 20
CHAPTER III **24**
CHARACTERISATION OF GROUP IV *ARABIDOPSIS* SULFURTRANSFERASES 24
Abstract 24
Introduction 25
Material & Methods 27
Plant material 27
DNA cloning techniques 27
Expression, extraction and purification of AtStr proteins in *E. coli* 28
SDS-PAGE and Western blot analysis 29
Transient expression of GFP fusion constructs in *Arabidopsis* protoplasts and microscopic analysis 30
Miscellaneous 30
Results 30
Identification and *in silico* characterisation of the putative *Arabidopsis* sulfurtransferase proteins of group IV 30
Expression and purification of recombinant *Arabidopsis* sulfurtransferases 33
Subcellular localisation of AtStr9, AtStr10 and AtStr11 34
Discussion 37
CHAPTER IV **42**
DIFFERENTIAL EXPRESSION OF *ARABIDOPSIS* SULFURTRANSFERASES UNDER VARIOUS CONDITIONS 42
Abstract 42

Introduction 42
Material & Methods 44
Growth, treatment and harvest of plants 44
RNA extraction and Northern blot analysis 45
Enzyme activity measurements 46
Analysis of ions and metabolites 46
Miscellaneous and statistical evaluation 47
Results 47
Nutritional influence on sulfurtransferase expression and activity 47
Expression levels and enzyme activity of sulfurtransferases during plant development 52
Expression levels and enzyme activity of sulfurtranferases during a diurnal light/dark cycle 54
Discussion 56
CHAPTER V 61
CHARACTERISATION OF *ARABIDOPSIS* SULFURTRANSFERASE T-DNA KNOCK-OUT MUTANTS AND TRANSGENIC *NICOTIANA* PLANTS OVEREXPRESSING *AtStr1* 61
Abstract 61
Introduction 62
Material & Methods 64
Growth, treatment and harvest of plants 64
Growth and maintenance of pathogenic bacteria and inoculation 65
PCR analysis of mutant plants 65
Southern blot analysis 66
SDS-PAGE and Western blot analysis 67
Enzyme activity measurements 67
Chlorate sensitivity test 67
Analysis of ions and metabolites 67
Results 68
Confirmation of a T-DNA insertion in the genome of *Arabidopsis* mutant plants 68
Phenotypical characterisation of *Arabidopsis* T-DNA insertion mutants 69
Sulfur metabolite contents in *Arabidopsis* T-DNA insertion mutants 70
Sulfurtransferase enzyme activity in *Arabidopsis* T-DNA insertion mutants 71
Chlorate sensitivity of *atstr15 Arabidopsis* T-DNA insertions mutants 72
Confirmation of *Arabidopsis AtStr1* in the genome of transgenic *Nicotiana* plants 73
Characterisation of transgenic *Nicotiana* lines overexpressing *AtStr1* 74
Sulfurtransferase enzyme activity in transgenic *Nicotiana* lines overexpressing *AtStr1* 75
Inoculation of transgenic *Nicotiana* lines overexpressing *AtStr1* with pathogenic bacteria 75
Discussion 77
CHAPTER VI 83
GENERAL DISCUSSION 83
Group I AtStr in a global Str context, focusing on structure and possible *in vivo* functions 83
Group IV AtStr in a global Str context, focusing on subcellular localisation and possible *in vivo* functions 85
Group VI AtStr in a global Str context, focusing on possible *in vivo* functions 87
General aspects on substrate specificity and conclusions 88
REFERENCES 91

CHAPTER I

General introduction

General and structural aspects of sulfurtransferase proteins

Sulfurtransferases (Str; EC 2.8.1.x) comprise a group of enzymes widely distributed in archaea, eubacteria and eukaryota, that catalyse the transfer of a sulfur atom from a suitable sulfur donor to a nucleophilic sulfur acceptor. The most studied and best characterised Str to date is the bovine liver rhodanese (Rhd) (thiosulfate:cyanide Str, EC 2.8.1.1), catalysing *in vitro* the transfer of a sulfane sulfur atom from thiosulfate (TS) to cyanide, leading to the formation of sulfite and thiocyanate (Westley, 1973). According to the generally accepted reaction mechanism (Fig. 1) the enzyme cycles between two different forms, the free enzyme and a sulfur-substituted intermediate (Rhd-S) during catalysis.

$$S_2O_3^{2-} + \text{Rhd} \rightarrow SO_3^{2-} + \text{Rhd-S}$$

$$\text{Rhd-S} + CN^- \rightarrow \text{Rhd} + SCN^-$$

Fig. 1 Reaction mechanism representing the sulfur transfer catalysed by thiosulfate:cyanide Str (EC 2.8.1.1).

Crystallographic investigations have shown that the Rhd-S intermediate is characterised by a persulfide bond at the sulfhydryl group of the essential cysteine (Cys) residue 247 in the active-site loop (Ploegman et al., 1979; Gliubich et al., 1996, 1998). 3-mercaptopyruvate (3-MP) Str (3-MP:cyanide Str, E.C. 2.8.1.2) catalyse the sulfur transfer to cyanide from 3-MP as a sulfur donor (Westley, 1973). A number of molecules can serve as sulfur donors in the Str reaction, such as TS and 3-MP, thiosulfonates and persulfides. Several compounds, such as cyanide, thiols, and dithiols, can act as sulfur acceptors. For a listing of the several types of Str enzymes, their respective possible substrates and proposed reactions catalysed, see Papenbrock (2002) or visit the enzyme nomenclature database (http://us.expasy.org/enzyme).

The structurally well-defined Rhd homology domain is the characteristic feature unifying all members of the Str / Rhd multi protein family. Rhd domains are ubiquitous structural modules that can be found as tandem repeats, with the C-terminal domain hosting the active-site Cys residue, as single domain proteins or in combination with distinct protein domains (Bordo & Bork, 2002). Sequence searches through completed genomes indicate that the *Escherichia coli* GlpE protein can be considered as the prototype structure for the ubiquitous

single-domain Rhd module (Spallarossa et al., 2001). The tertiary structure of bovine Rhd is composed of two globular domains of nearly identical size and conformation, connected by a linker sequence. Although the three-dimensional fold of the two domains is quite similar the overall sequence homology is very low (Ploegman et al., 1978). *In silico* studies show that Rhd domains are structurally related to the catalytic subunit of Cdc25 phosphatase and arsenate reductase enzymes (Hofmann et al., 1998). The recognition and specificity for substrates is highly affected by the amino acid composition of the active-site loop (Bordo & Bork, 2002). By mutagenic elongation of the active-site loop by one additional amino acid, the substrate specificity of *Azotobacter vinelandii* RhdA could be changed from sulfate to phosphate containing compounds (Forlani et al., 2003).

In the *Arabidopsis* genome 18 genes encoding Str-like proteins (AtStr) each containing at least one Rhd domain have been identified by database searches (Bauer & Papenbrock, 2002). Recently, two further putative Str proteins displaying homology to AtStr4 and AtStr17 have been found additional to the 18 already identified (AtStr4a, AtStr17a). The proteins have been divided into six groups according to amino acid sequence homologies (Table 1). So far two two-domain Str proteins preferring 3-MP over TS *in vitro* (Nakamura et al., 2000, Papenbrock & Schmidt, 2000a, b) and two single-domain TS specific Str, resembling the C-terminus of the *Arabidopsis* two-domain AtStr1 and the single-domain GlpE protein from *E. coli*, could be isolated and characterised in *Arabidopsis* (Bauer & Papenbrock, 2002). The linker sequence connecting the two domains in plant two-domain Str is exceptionally longer than in other species. The removal of the plant specific prolongation of the linker sequence from AtStr1 led to a reduction of enzyme activity to about 30% and drastically reduced the stability of the protein towards increased temperature and urea treatment. Besides a role in stabilisation and activity of the protein, a role of the hydrophobic linker sequence in substrate binding was suggested. The elongation may provide an extended hydrophobic environment to bind larger substrate molecules, such as proteins (Burow et al., 2002), as proposed also for the bovine two-domain Rhd (Luo et al., 1995). However, the true biological role of the plant specific prolongated interdomain linker sequence has not been unambiguously clarified yet.

Besides C332, identified as the catalytically important Cys residue, binding the sulfur to be transferred in a persulfide bond during catalysis, the amino acid sequence of AtStr1 contains four further Cys residues. At least C339 in close vicinity to the catalytic C332 is believed to have some impact on the sulfur transfer reaction (Burow et al., 2002). In which way the C339 takes part in the transfer reaction is yet to be determined.

	gene ID	No. aa	localisation (pred/exp)	reference or source	miscellanous remarks	reference or source
Group I						
1	At1g79230	322	MT (exp)	Bauer et al., 2004 Hatzfeld & Saito, 2000 Heazlewood et al., 2004 Nakamura et al., 2000 Papenbrock & Schmidt, 2000a	thiosulfate sulfurtransferase 3-mercaptopyruvate sulfurtransferase	Hatzfeld & Saito, 2000 Nakamura et al., 2000 Papenbrock & Schmidt, 2000a, b
2	At1g16460	318	Cyt (exp)	Bauer et al., 2004 Hatzfeld & Saito, 2000 Nakamura et al., 2000	thiosulfate sulfurtransferase 3-mercaptopyruvate sulfurtransferase	Hatzfeld & Saito, 2000 Nakamura et al., 2000 Papenbrock & Schmidt, 2000b
Group II						
3	At5g23060	387	MT (exp)	Heazlewood et al., 2004	similar to unknown protein	db annotation
4	At4g01050	457	CP, thylakoid membrane (exp)	Peltier et al., 2004	hypothetical protein	db annotation
4a	At3g25480	264	CP, thylakoid membrane (exp)	Peltier et al., 2004	hypothetical protein	db annotation
Group III						
5	At5g03455	132	nucleus (pred)	Landrieu et al., 2004	dual-specificity tyrosine phosphatase putative Acr2 arsenate reductase	Landrieu et al., 2004 Duan et al., 2005
6	At1g09280	581	Cyt (pred)	db annotation	At1g09280/T12M4_1	db annotation
7	At2g40760	522	MT (pred)	db annotation	unknown protein	db annotation
8	At1g17850	366	CP/ER (pred)	db annotation	contains Rhodanese-like PF|00581 domain	db annotation
Group IV						
9	At2g42220	234	CP, thylakoid membrane (exp)	Peltier et al., 2004 this work, chapter III	*Datura innoxia* homolog Cd^{2+} induced	Louie et al., 2003
10	At3g08920	214	MT (pred/preliminary exp)	this work, chapter III	unknown protein	db annotation
11	At4g24750	260	CP/Per (pred/preliminary exp)	this work, chapter III	putative protein	db annotation
Group V						
12	At5g19370	309	CP/MT (pred)	db annotation	putative peptidyl-prolyl cis-trans isomerase	db annotation
13	At5g55130	464	CP/Cyt (pred)	db annotation	Molybdopterin synthase sulphurylase; SIR1, negative regulation in auxin signalling	db annotation Zhao et al., 2003
Group VI						
14	At4g27700	237	CP (exp)	Bauer et al., 2004 Peltier et al., 2004	hypothetical protein	db annotation
15	At4g35770	182	CP, thylakoid membrane (exp)	Bauer et al., 2004	AtSen1, senescence association, dark-induced	Oh et al., 1996 Schenk et al., 2005
16	At5g66040	120	CP (exp)	Bauer et al., 2004	senescence-associated protein sen1-like protein; ketoconazole resistance protein-like thiosulfate sulfurtransferase	db annotation Bauer & Papenbrock, 2002
17	At2g17850	150	nucleus (pred)	db annotation	putative senescence-associated rhodanese protein; similarity to Ntdin homolgy to defense and stress associated *Curcubitaceae* proteins	Yang et al., 2003 Walz et al., 2004
17a	At2g21045	169	Cyt (pred)	db annotation	senescence-associated protein	db annotaion
18	At5g66170	136	Cyt (exp)	Bauer et al., 2004	senescence-associated protein sen1-like protein thiosulfate sulfurtransferase	db annotaion Bauer & Papenbrock, 2002

Table 1 Overview of the 20 putative Str in *Arabidopsis thaliana*. The protein name, gene identification, number of amino acids, predicted or experimentally shown localisation and remarks on different aspects are summarised including the respective references. The programs Predotar, PSORT, and TargetP were used for the localisation prediction (http://www.expasy.ch/tools). The respective probabilities are given. Abbreviations: aa, amino acids; CP, chloroplast; db, database; ER, endoplasmatic reticulum; exp, experimental; ID, identification; kDa, kilo Dalton; MT, mitochondrial; MW, molecular mass; no., number; PER, peroxisome; pred, predicted.

Subcellular localisation of sulfurtransferase proteins

In the organisms analysed so far, Str proteins have been identified in different subcellular compartments. In *E. coli* the single-domain GlpE protein was localised in the cytoplasm while a two-domain Str was located in the periplasm (Ray et al., 2000). A Rhd-like protein in *Synechococcus* was also found in the periplasmic space and suggested to be involved in transport processes (Laudenbach et al., 1991). In rats two distinct Str proteins displaying a substrate preference for 3-MP or TS, respectively, were identified. While the 3-MP Str was located in cytoplasm and mitochondria the TS Str was found only in mitochondria. The proteins have been suggested to detoxify cyanide in the cytoplasm (3-MP Str) and to protect cytochrome c oxidase in mitochondria (Nagahara et al., 1995, 1999). A tobacco homologue of AtStr15, Ntdin, was recently identified in chloroplasts (Yang et al., 2003). In *Arabidopsis* two-domain Str proteins have been localised in mitochondria (AtStr1) and in the cytoplasm (AtStr2) by applying different methods (Bauer et al., 2004; Hatzfeld & Saito, 2000, Nakamura et al., 2000; Papenbrock & Schmidt, 2000a). Of the four single-domain AtStr analysed so far, three proteins are localised in chloroplasts (AtStr14, AtStr15 thylakoid membrane, AtStr16) while AtStr18 was shown to be cytoplasmic (Bauer et al., 2004). Recently, AtStr4, AtStr4a, AtStr9 and AtStr14 were identified as being part of the thylakoid membrane proteome in *Arabidopsis* by three phase partitioning and mass spectrometry (Peltier et al., 2004). The localisation of the other members of the AtStr multi protein family is yet unknown and remains to be determined.

The knowledge of the subcellular localisation of the proteins can provide valuable hints for the elucidation of the metabolic or regulatory pathway(s) in which the proteins are active and of the biological function(s) of the respective proteins in the plant organism.

Putative *in vivo* functions of sulfurtransferase proteins

Although Str proteins are ubiquitously present in all three phyla, their function is still largely debated. Since the *in vivo* substrates of the proteins have not been unambiguously identified thus far, specific biological roles for most members of the Str / Rhd homology superfamily have not been verified yet (Spallarossa et al., 2001).

Different functions have been proposed: the detoxification of cyanide (Nagahara et al., 1999; Vennesland et al., 1982) and reactive oxygen species (Nandi et al., 2000), an involvement in sulfate assimilation (Donadio et al., 1990), mobilisation of sulfur for iron-sulfur cluster biosynthesis or repair (Bonomi et al., 1977), sulfur transfer for the biosynthesis of thiamin, thiouridine and molybdenum cofactor (MoCo) (Lauhon & Kambampati, 2000; Leimkühler et al., 2001; Palenchar et al., 2000; Yang et al., 2003) and an involvement in transport processes of reduced sulfur (Laudenbach et al., 1991; Papenbrock & Schmidt, 2000b). Rhd domain proteins have been associated with specific stress conditions (Bordo & Bork, 2002). Since the reduced sulfur transferred is biochemically labile a regulatory impact of the sulfur transfer in biological systems was proposed (Toohey, 1989). Furthermore, the frequently observed association of catalytically inactive Rhd modules with other protein domains suggests a distinct role of these inactive domains, possibly in regulation and/or signalling (Bordo & Bork, 2002). Thioredoxin was shown to serve *in vitro* as sulfur acceptor of the recombinant leishmanial LmajMST and has been suggested to be the physiological sulfur acceptor in the reaction catalysed by the *E. coli* GlpE (Ray et al., 2000; Williams et al., 2003). The recombinant mammalian Rhd expressed in *E. coli* also can catalyse the oxidation of reduced thioredoxin evidently by reactive oxygen species besides catalysing the sulfur transfer reaction. One isoform of the protein thus might act in detoxifying free oxygen radicals in mitochondria (Nandi et al., 2000). Interestingly, it could be shown that a selenium-substituted Rhd could serve as selenium donor in the *in vitro* selenophosphate synthetase assay. Selenophosphate is the active selenium donor required for the specific synthesis of SeCys-tRNA, the precursor of selenocysteine in selenoenzymes, in bacteria and mammals (Ogasawara et al., 2001). Very recently, also for the *E. coli ybbB* gene product a role in selenium metabolism was proposed (Wolfe et al., 2004). These results give rise to completely new possible functions of Str *in vivo*.

While mammalian rhodanese may be involved in the elimination of toxic cyanogenic compounds (Nagahara et al., 1999; Vennesland et al., 1982), the evidence for an

involvement of plant Str in cyanide detoxification is rather low (Chew, 1973; Kakes & Hakvoort, 1992; Meyer et al., 2003) but can not be fully excluded yet. Results concerning a function in providing reduced sulfur for iron-sulfur cluster synthesis are contradictory. Specific plant Str might be involved while others might not (Nakamura et al., 2000; Pagani et al., 1984). Expression of *AtStr1* and *AtStr2* and Str activity increased during senescence of plants. A role of these Str proteins in the mobilisation of sulfur for transport processes from older leaves to younger tissues during senescence was suggested (Papenbrock & Schmidt, 2000b). Also for other AtStr an association to senescence and to stress was postulated (Meyer et al., 2003; Papenbrock & Schmidt, 2000b). In radish, tobacco and *Arabidopsis* dark-inducible, senescence-associated Str proteins encoded by *Rsdin1*, *Ntdin* and *AtSEN1* (*AtStr15*) were identified and characterised (Chung et al., 1997; Oh et al., 1996; Shimada et al., 1998; Yang et al., 2003). Of these the *Ntdin* gene product was recently shown to be involved in MoCo biosynthesis (Yang et al., 2003). In *Datura innoxia* a cadmium-induced AtStr9 homologue was identified, suggesting a role of the Str in reaction to heavy metal stress (Louie et al., 2003). Furthermore, in wheat a Str might be involved in resistance against the fungal pathogen *Erysiphe graminis* (Niu et al., 2002).

The high abundance of putative Str proteins in *Arabidopsis*, the variance in the amino acid composition of the active-site loop that is crucial in substrate recognition and catalytic activity (Bordo & Bork, 2002; Forlani et al., 2003), and the localisation of Str in different compartments of the cell (Bauer et al., 2004; Hatzfeld & Saito, 2000; Nakamura et al., 2000; Papenbrock & Schmidt, 2000a) suggest an involvement of the 20 putative AtStr proteins in distinct biological functions. The analysis of transgenic plants represents a useful tool in providing knowledge on the possible function(s) of Str proteins in the plant organism.

Aims and experimental program of this thesis

- Further investigations on the role of the prolonged interdomain linker sequence and of the two most important Cys residues in AtStr1 on conformation, stability and enzyme activity by spectroscopic analyses

- Heterologous expression of the three *AtStr* genes of group IV in *E. coli*, purification and characterisation of the *in vitro* enzyme activities of the recombinant proteins

- Identification of the best suitable donor and acceptor substrates of the three AtStr of group IV *in vitro* and *in vivo*

- Determination of the subcellular localisation of the three AtStr proteins of group IV by transient transformation of fusion constructs with the green fluorescent protein (GFP)

- Functional analysis of several AtStr proteins by determination of expression patterns and sulfur metabolite profiling under various growth conditions

- Characterisation of *Arabidopsis* T-DNA insertion mutants and generation and characterisation of transgenic *Nicotiana* plants heterologously overexpressing *AtStr1*

CHAPTER II

Conformational studies on *Arabidopsis* sulfurtransferase AtStr1 analysed by spectroscopic methods

Abstract

Sulfurtransferases/rhodaneses (Str) are a group of enzymes widely distributed in archaea, prokaryota and eukaryota that catalyse the transfer of sulfur from a donor molecule to a thiophilic acceptor substrate. In this reaction Str cycles between the sulfur-free and the sulfur-substituted form. Two-domain Str consist of two globular domains of nearly identical size and conformation connected by a short linker sequence which is elongated in plant two-domain Str proteins compared to Str in other organisms. The two-domain *Arabidopsis* Str1 protein (At1g79230) was expressed in *E. coli* as mature protein, and in mutated forms without the elongated linker sequence and as AtStr1C332S and AtStr1C339V. The persulfuration state of the purified recombinant proteins was investigated in the presence and absence of sulfur donors by fluorescence spectroscopy. To obtain information about the percentages of the proteins' secondary structure circular dichroism (CD) in the far UV range was conducted. The overall changes in tertiary structure were determined by CD in the near UV range. Finally, the proteins' stability was analysed by tryptic digestion. The elongated linker sequence is essential for the correct conformation and the stability and thereby also affects the catalytic activity of the AtStr1 protein. The replacement of the catalytic cysteine residue C332 leads to higher rigidity of the molecule whereas the replacement of C339 does not lead to any conformational changes supporting a proposed involvement of C339 in catalysis.

Introduction

Sulfurtransferase (Str) enzymes catalyse the transfer of a sulfur atom from suitable sulfur donors to nucleophilic sulfur acceptors. The most studied and best characterised Str is bovine rhodanese (Rhd) (thiosulfate:cyanide Str, EC 2.8.1.1) which catalyses, *in vitro*, the transfer of a sulfane sulfur atom from thiosulfate (TS) to cyanide, leading to the formation of sulfite and rhodanide (Westley, 1973). Solution studies have identified three catalytic requirements: an active site sulfhydryl group, two cationic residues, and a hydrophobic environment for substrate binding (Finazzi Agro et al., 1972; Miller-Martini et al., 1994a,

1994b). However, *in vivo* neither donor substrates nor sulfur acceptors could be clearly identified in any of the organisms investigated.

By using different strategies to mine the databases Str-like proteins containing typical Rhd patterns or domains could be identified in *Arabidopsis* (Bauer & Papenbrock, 2002; Bartels & Papenbrock, unpublished results). Proteins containing Rhd domains are structural modules found as single-domain proteins, as tandemly repeated modules in which only the C-terminal domain bears the properly structured active site, or as members of multi domain proteins. Two of the *Arabidopsis* Str, AtStr1 and AtStr2, belong to the two-domain Str consisting of two Rhd domains (N- and C-terminal) of nearly identical size connected by a linker sequence. This overall structure is similar to the structure of bovine Rhd; however, in plant two-domain Str the connecting linker sequence is elongated in comparison to the linker of other two-domain sequences from eukaryotic organisms (Papenbrock & Schmidt, 2000b). The role of the elongated linker could not be clarified unambiguously so far. The AtStr1 sequence contains five cysteine (Cys) residues of which the catalytic Cys residue C332 is the target of persulfide formation. In plant two-domain Str a second Cys (C339) is located close to the catalytic Cys; its function in catalysis and conformational stability has not been clarified yet.

Despite the presence of Str/Rhd activities in all three domains of life, the physiological roles of the members of this multi protein family have not been established unambiguously. Proposed roles include cyanide detoxification (Vennesland et al., 1982), sulfur metabolism (Donadio et al., 1990), and mobilisation of sulfur for iron-sulfur cluster biosynthesis or repair (Bonomi et al., 1977; Pagani et al., 1984). In plants also the mobilisation of sulfur for transport processes in older leaves was suggested (Papenbrock & Schmidt, 2000b).

Obligatory intermediates in Str/Rhd catalysis, in the presence of the donor TS, are E (sulfur-free enzyme) and ES (sulfur-substituted enzyme). In the reaction Rhd cycles between the E and ES form. The intrinsic fluorescence of Rhd resulting from tryptophan residues is quenched when a stable persulfide is formed in ES due to energy transfer between the persulfide and the initially excited tryptophan residues and can be followed by fluorescence spectroscopy.

Circular dichroism (CD) relies on the differential absorption of left and right circularly polarised radiation by chromophores which either possess intrinsic chirality themselves or are placed in chiral environments. Proteins contain a number of chromophores which can give rise to CD signals. In the far UV region (180 to 240 nm) the absorbing band is

principally the peptide bond. Far UV CD analysis can be used to quantitatively assess the overall secondary structure content of the protein because the different forms of regular secondary structure found in proteins generate different spectra (Kelly & Price, 2000). The near UV CD detects the aromatic amino acids constrained in rigid chiral environments, revealing their embedding in tertiary structure (Adler et al., 1973). Thus, CD is an ideal technique to monitor conformational changes in proteins which can occur as a result of changes in experimental conditions or caused by mutations (Kelly & Price, 2000).

The aim of this study was to investigate the role of the unique plant linker sequence and the two most important out of five Cys residues in AtStr1. Recombinant mutated proteins were expressed, purified and analysed by different spectroscopic methods in comparison to the mature wild-type protein.

Material & Methods

Expression and purification of the AtStr proteins in *E. coli*

The AtStr1 protein, the mutants AtStr1C332S and AtStr1C339V, the AtStr1 protein excluding plant specific linker sequence elongation (AtStr1wlink) and the N- and the C-terminal domain of the AtStr1 protein were expressed and purified as described (Burow et al., 2002).

Investigations of the proteins by fluorescence spectroscopy

Fluorescence measurements were performed using an LS50 spectrofluorimeter (Perkin Elmer) equipped with a thermostatic cell holder at 20°C. The excitation wavelength was 280 nm in all experiments, slit width for excitation and emission were 5 and 3 nm, respectively. Proteins were used at a concentration of 3 µM in 20 mM Tris/HCl, pH 8.0. Emission spectra were scanned from 300 to 400 nm 1 min after reagent addition and were corrected for dilutions due to reagent addition. The fluorescence intensities observed at 336 nm (F_{obs}) are presented as ΔF(%) defined as:

$$\Delta F(\%) = -\frac{F_{obs} - F_0}{F_0} \times 100$$

where F_0 is the original fluorescence intensity of the analysed protein as described by (Colnaghi et al., 2001).

Structural analysis of proteins by circular dichroism

Far UV CD spectra were recorded at 25°C in a Jasco J-810 spectropolarimeter (Great Dunmow, UK) at a scan rate of 10 nm x min^{-1}, a response of 0.25 s, and a bandwidth of 1 nm. Analyses were performed at a protein concentration of 3 µM in 20 mM Tris/HCl, pH 8.0. Data were collected at 0.1 nm intervals in 0.01 cm quartz cells from 190 to 260 nm. Recorded spectra were corrected for the buffer baseline. At least three accumulations were done for each spectrum.

Limited proteolysis

Affinity purified proteins (1 µg x $µl^{-1}$) in 20 mM Tris/HCl, pH 8.0 were proteolysed with 0.5% trypsin (Serva, Heidelberg, Germany) at 23°C. The assay also contained 2 mM $CaCl_2$ to avoid autolysis of trypsin. The proteolysis was monitored by SDS-PAGE over a time scale of 60 min. The digestion was stopped at the time points indicated by adding 9 µl 4x SDS-PAGE sample buffer (2.4 ml mercaptoethanol, 6 ml 40% SDS, 240 µl bromophenol blue, 8 ml glycerol, 3.6 ml 1 M Tris/HCl, pH 6.8 in a total volume of 20 ml) to 15 µl aliquots of the assay and boiling the samples for 10 min. SDS-PAGE was done according to Laemmli (1970) using 15% acrylamide gels. Gels were stained with Coomassie brilliant blue or used for Western blot analysis.

Western blot analysis

Following SDS-PAGE proteolysed proteins were transferred to nitrocellulose membranes (Roth) by the semi-dry method and immobilised for immunodetection. Western blot analysis was done according to Sambrook et al. (1989) using a RGS antibody from mouse (Qiagen), anti-mouse Ig from rabbit (Sigma) and anti-rabbit antibody conjugated with alkaline phosphatase (Sigma). Colorimetric detection was carried out using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate.

Miscellaneous

Protein concentrations were either determined according to Bradford (1976) using bovine serum albumin (Roth) as a protein standard or spectrophotometrically at 280 nm using the extinction coefficient following formula 1.55 x A_{280} resulting in the protein concentration in µg x $µl^{-1}$ (Layne, 1957). The amino acid sequence analyses and prediction of the molecular masses were performed with the computer program Protean in DNASTAR (Madison, WI, USA).

Results

Status of persulfuration and the influence of substrates

Sulfur transfer catalysis does imply the formation of a persulfurated intermediate form of the Str enzyme (Finazzi Agro et al., 1972; Pagani et al., 2000). The formation of the covalent enzyme-sulfur intermediate can be detected by the intrinsic fluorescence quenching of the enzyme due to energy transfer between the persulfide and the initially excited tryptophan residues (Horowitz et al., 1983; Cannella et al., 1986). The fluorescence spectrum of purified AtStr1 changed following the addition of KCN (Fig. 1A). This nucleophilic acceptor could remove the sulfane sulfur from the persulfide bond to the catalytic Cys. The observed quenching of fluorescence indicates that the recombinant AtStr1 full-length protein was isolated in a stable persulfurated form.

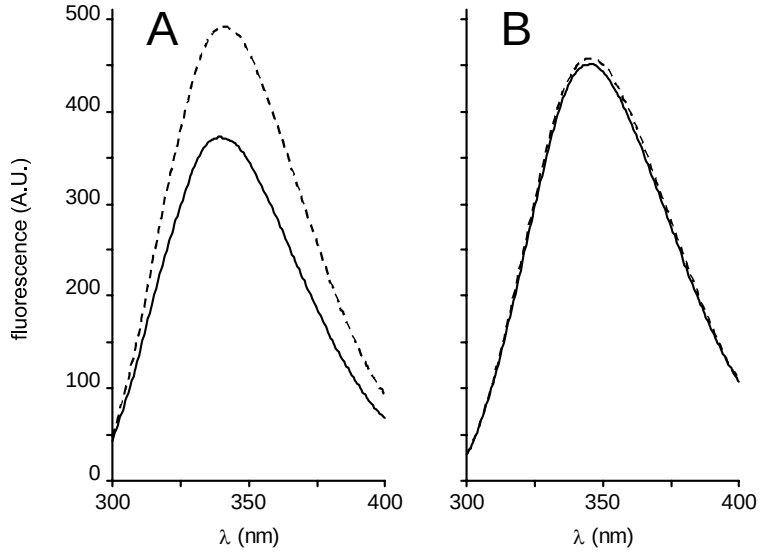

Fig. 1 Influence of the AtStr1 linker on protein persulfuration state. Fluorescence spectra (excitation at 280 nm) of 3 µM purified AtStr1 (panel A) and AtStr1wlink (panel B) in 20 mM Tris/HCl pH 8.0 before (full line) and after addition of 10-fold molar excess of KCN (dashed line). Spectra were corrected for dilution due to KCN addition. A.U., fluorescence arbitrary units.

The AtStr1 protein showed Str activity *in vitro* using 3-mercaptopyruvate (3-MP) and TS as sulfur donor substrates. The activity with TS was about six fold less, however, the K_m value for TS was lower than that for 3-MP (Papenbrock & Schmidt, 2000a; Burow et al., 2002). There is an obvious contradiction in the behaviour of 3-MP and TS in the affinity to and binding by Str compared to their utilisation as sulfur donor substrates. Therefore the ability of 3-MP and TS to generate the sulfur-substituted form of AtStr1 was monitored by determining the intrinsic fluorescence changes as a function of stoichiometric addition of the donor substrates (Fig. 2A and 2B). Both substrates were able to generate the sulfur-substituted form of AtStr1. Titration analysis revealed that TS showed higher affinity to

AtStr1 as compared to 3-MP (data not shown). This result is in agreement with the published K_m values (Burow et al., 2002).

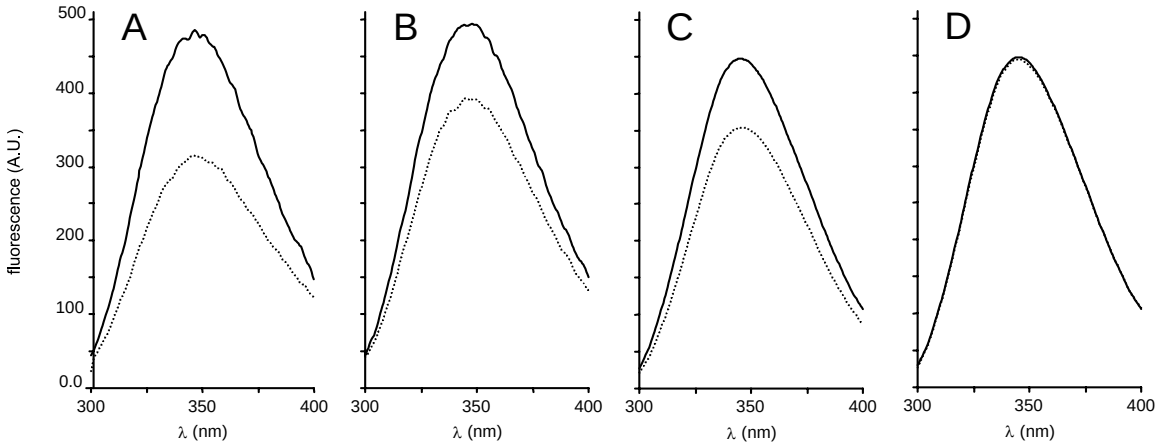

Fig. 2 Sulfur donor ability for AtStr1 and AtStr1wlink persulfuration. Fluorescence spectra (excitation at 280 nm) of 3 µM AtStr1 (panel A and B) and AtStr1wlink (panel C and D) in 20 mM Tris/HCl pH 8.0 before (full line) and after (dotted line) addition of 10-fold molar excess of 3-mercaptopyruvate (panel A and C) or thiosulfate (panel B and D). Both proteins were sulfane-sulfur deprived by cyanide treatment and excess reagent was removed by gelfiltration before the fluorescence analysis. Spectra were corrected for dilution due to sulfur donor addition. A.U., fluorescence arbitrary units.

The catalytic Cys residue C332 is the target of persulfide formation (Ploegman et al., 1979; Horowitz & Criscimagna, 1983). Close to this Cys a second Cys (C339) is located in plant two-domain Str. Each Cys residue was replaced by serine or valine, respectively, to investigate their impact on the persulfuration status. Formation of the persulfurated intermediate is mandatory for sulfur transfer catalysis, and, as expected, in AtStr1C332S the lack of TS and 3-MP Str activities (Burow et al., 2002) paralleled the complete absence of persulfuration by TS and 3-MP. Enzyme activities of AtStr1C339V were reduced to about 80% with 3-MP and to about 25% with TS. Different protein preparations of AtStr1C339V showed different results: Either the protein was persulfurated directly after purification as indicated by increased fluorescence after addition of KCN or it could be persulfurated by 3-MP or TS, or it could not be persulfurated at all. Therefore it is difficult to draw conclusions about the role of C339 during catalysis. The choice of the exchanged amino acid might have an impact because valine is of bigger size than Cys and there could be steric alterations influencing the capability to bind 3-MP as substrate which is of bigger size than TS. On the other hand, enzyme activity measurements showed the protein was active with 3-MP used as substrate (Burow et al., 2002).

Role of the linker: results of fluorescence spectroscopy

In all two-domain Str identified so far the N- and C-terminal domains are connected by a linker sequence. In plant two-domain Str sequences the connecting linker sequence between both domains is around 20 amino acids longer than in various Str sequences from prokaryotic and other eukaryotic species. To obtain more information about the function of this linker sequence a peptide of 23 amino acids was deleted from AtStr1 (AtStr1wlink). This deletion halved the specific enzyme activity with 3-MP in comparison to the specific activity of wild-type AtStr1. When TS was used the AtStr1wlink protein displayed only about one third of the wild-type activity (Burow et al., 2002). It was also shown previously, that the activity of the mature AtStr1 protein including the elongated linker sequence was less affected by thermal denaturation and treatment with 4 M urea than the recombinant protein without the linker (Burow et al., 2002). In this work we investigated the AtStr1 protein without the linker elongation (AtStr1wlink) by spectroscopic methods in more detail. The fluorescence spectra show that the ability of AtStr1wlink to form a stable persulfide group is almost completely lost (Fig. 1B). In addition, fluorescence experiments indicate that the formation of the persulfide group was not achieved in the presence of TS (Fig. 2D) but only in the presence of 3-MP (Fig. 2C) in contrast to results obtained with AtStr1 (Fig. 2A and 2B).

Role of the linker: results obtained by circular dichroism spectroscopy analysis in the near UV range

In order to get deepen insights on the effect of the removal of the elongated linker peptide we analysed the conformational state of AtStr1wlink by CD spectroscopy in comparison to the wild-type and the two mutated AtStr1 proteins (Fig. 3). Any decrease of a near UV CD signal indicates changes in the position of the aromatic amino acids tryptophan (signals at about 290 nm), tyrosine (signals at about 275 to 283 nm), and phenylalanine (signals at about 255 to 270 nm) at the surface of proteins and reflects a reduction in the order of tertiary structure. Also the formation of disulfide bridges produces a weak signal at 260 nm which is often masked by signals of aromatic residues (Pouvreau et al., 2005).

The mature AtStr1 contains nine tryptophan residues mainly at the N-terminus (six out of nine), eight tyrosine residues, 12 phenylalanine residues and five Cys residues. AtStr1wlink contains one tyrosine residue less; the effects of the missing tyrosine residue are not predictable, but probably negligible. AtStr1 displays positive bands at 294, 288, 279, 272, 265, and 260 nm (Fig. 3). AtStr1C332S displays a sharper profile in the range of

positive bands at 294 and 287 nm indicating a more rigid conformation of the tertiary structure in the regions of tryptophan residues. AtStr1C339V possesses a modified weakened tertiary structure relative to the other proteins. The signals are flat especially in the tyrosine-tryptophan region of the spectrum. The signal might be low because in the region of 280 to 300 nm the signal can not be well resolved indicating the co-presence of multiple conformations due to absence of rigidity, high flexibility or instability of the structure (Fig. 3).

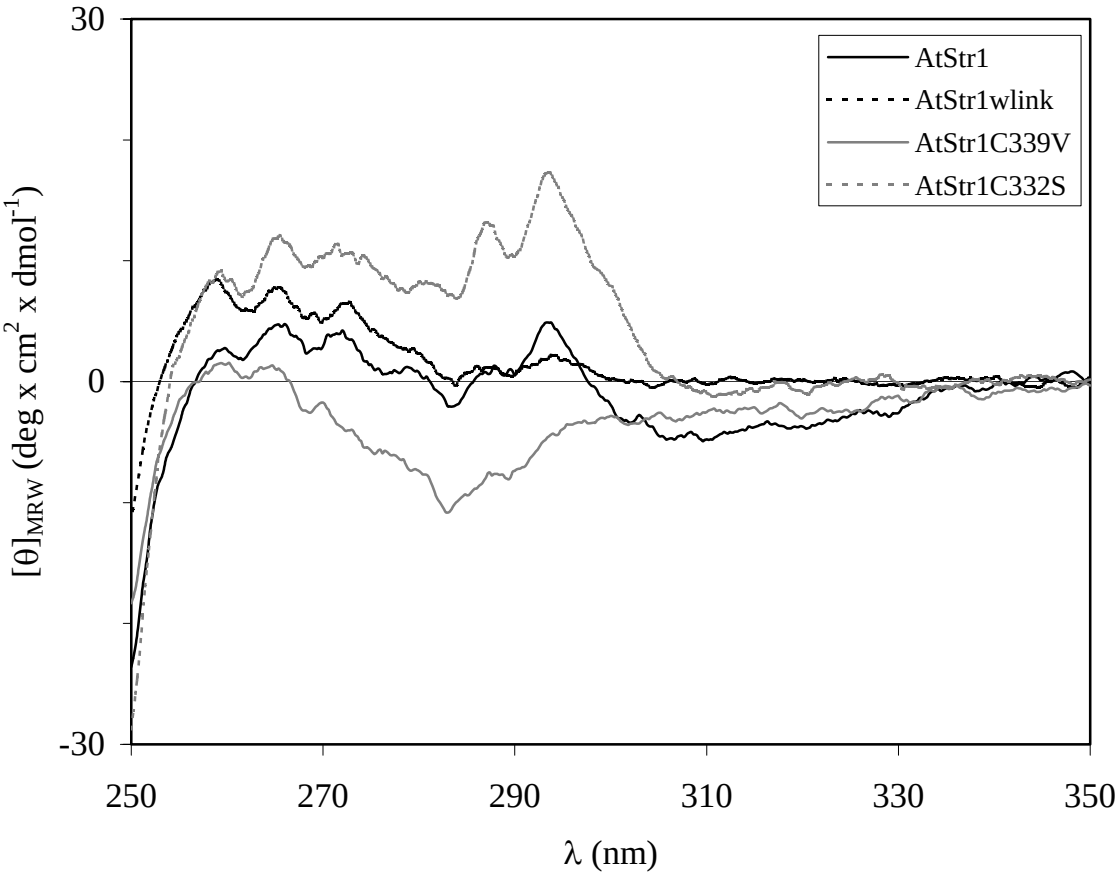

Fig. 3 Near UV CD spectra of AtStr1 with and without linker elongation, and AtStr1C332S and AtStr1C339V. Purified proteins in 20 mM Tris/HCl pH 8.0. AtStr1 1.012 µg protein x μl^{-1} (bold filled line); AtStr1wlink 0.679 µg protein x μl^{-1} (bold dotted line); AtStr1C332S 0.789 µg protein x μl^{-1} (filled line); AtStr1C339V 0.781 µg protein x μl^{-1} (dotted line). Data deconvolution was done using CDNN 2.1. Spectra were recorded 33-fold and averaged.

The spectrum of AtStr1wlink shows a decrease of the 294 nm signal relative to AtStr1. The loss of signals from tryptophan residues indicates that the linker of AtStr1 is important to maintain a compact conformational structure. The CD measurements also showed that the signal of AtStr1wlink was nearly zero in the range from 283 to 350 nm suggesting that the lack of the linker peptide does not allow the acquisition of the native conformation. However, the overall structure of AtStr1wlink seems to be conserved since there is little

change in the phenylalanine spectrum. Because the proteins compared have the same number of aromatic amino acids, except AtStr1wlink, the differences measured in the spectra can be attributed to the altered environment of the aromatic amino acid residues.

Role of the linker: results obtained by circular dichroism spectroscopy in the far UV range

CD in the far UV range can give information about the percentages of the secondary structures of a protein. The spectra recorded between 190 and 260 nm revealed differences in the curve shape of AtStr1wlink in comparison to the three other proteins (Fig. 4). The spectra of these three proteins are characterised by a positive signal below 200 nm, a minimum at 208 nm, and a shoulder at 225 nm indicating that they have adopted an ordered structure according to Buhot et al. (2004) whereas the spectrum of AtStr1wlink does not show a minimum at 208 nm and no shoulder at 225 nm.

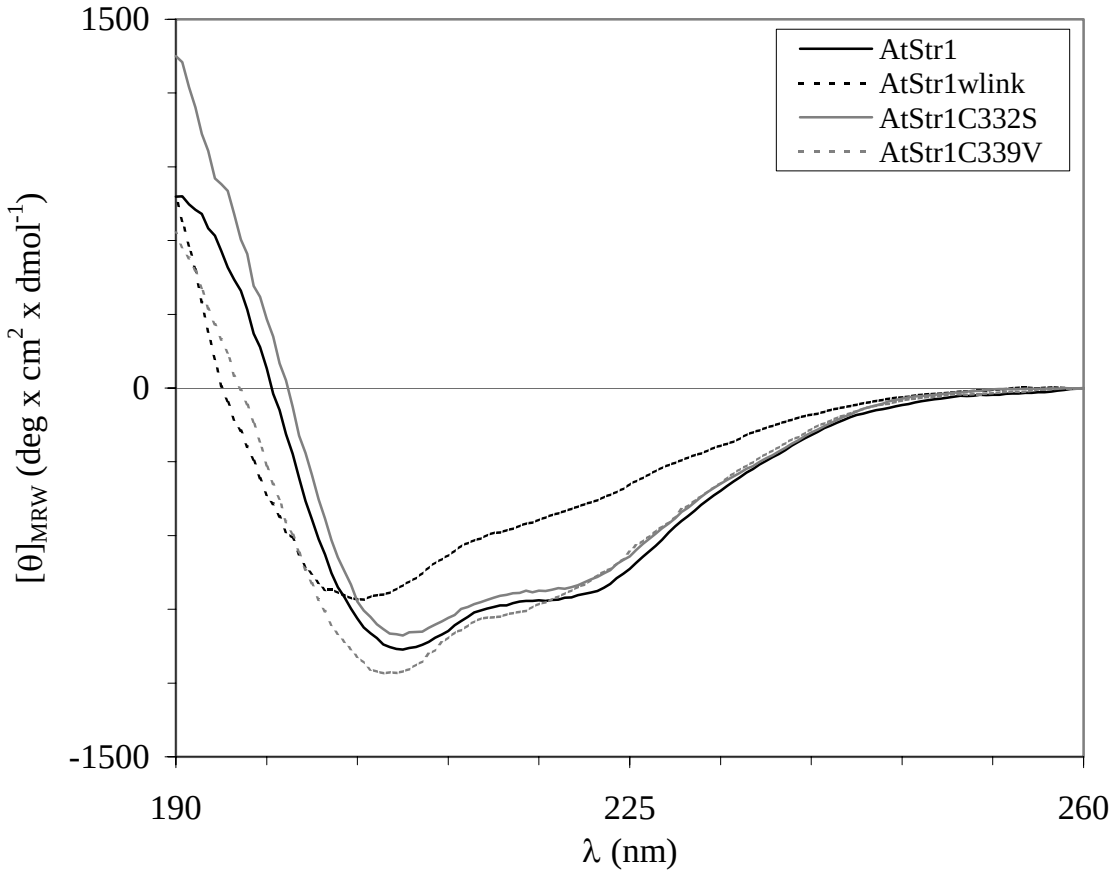

Fig. 4 Far UV CD spectra of AtStr1, AtStr1wlink, AtStr1C332S, and AtStr1C339V were recorded in 20 mM Tris/HCl pH 8.0. Concentration and different number of amino acids were taken in consideration. The different spectra of AtStr1, AtStr1wlink, AtStr1C332S, and AtStr1C339V are displayed with different lines as indicated in the small legend.

The calculated helical contents are very similar for AtStr1, AtStr1C332S and AtStr1C339V (27%) in agreement with the helical content calculated from the three-dimensional

structure of *Azotobacter vinelandii* RhdA (Bordo et al., 2000) while the helical content of AtStr1wlink was significantly lower (20%).

The application of a computer program for the prediction of the protein secondary structures (GORIV, Garnier et al., 1996) reveals that the 16 amino acids in the core of the elongated linker in plant two-domain Str with a high probability form the two-dimensional structure of an α-helix. This prediction could be confirmed experimentally: The C-terminal domain of the AtStr1 protein was expressed and purified including and excluding the extended linker sequence and the results of CD spectroscopy in the far UV range demonstrate a higher helix content in the C-terminus including the linker in comparison to the C-terminus alone (data not shown). Extended BLAST searches could not identify any sequence similarities of the plant linker sequence with other proteins.

Partial tryptic proteolysis

As CD spectroscopy revealed altered conformations of the recombinant AtStr1 mutants investigated, the consequences on the susceptibility to proteolytic digestion was analysed. In previous experiments the sensitivity to thermal denaturation and incubation with urea was determined only by activity measurements (Burow et al., 2002). The application of this third method might reveal whether wild-type and mutant proteins differ in their accessibility to proteolysis due to conformational changes caused by the mutations. Trypsin digests proteins specifically at the carboxy terminus of the basic amino acids lysine and arginine. AtStr1 contains 17 lysine and 14 arginine residues, therefore an almost uncountable number of fragments even after partial tryptic proteolysis is expected. AtStr1wlink contains only 15 lysine and also 14 arginine residues, therefore almost the same number of peptide fragments is expectable.

One µg of each protein preparation, AtStr1, AtStr1wlink, AtStr1C339V and AtStr1C332S, was digested by trypsin. At different time points, 0, 15, 30, 45, and 60 min, samples were taken plus a control at 60 min of the assay without trypsin and analysed by SDS-PAGE and Western blot analysis (Fig. 5). In the concentration used the trypsin band was not visible in the Coomassie-stained gels. To exclude any cross-reactivity of the RGS antibody with trypsin a 2 µg trypsin aliquot, about 40 times higher concentrated than used for digestion, was loaded on an SDS-gel and analysed by Western blotting. The 24 kDa trypsin band was not recognised by the RGS antibody (data not shown). Since all four recombinant proteins analysed contained a His_6-tag at the N-terminus, only the N-terminal part including the His_6-tag can be detected by the RGS-antibody.

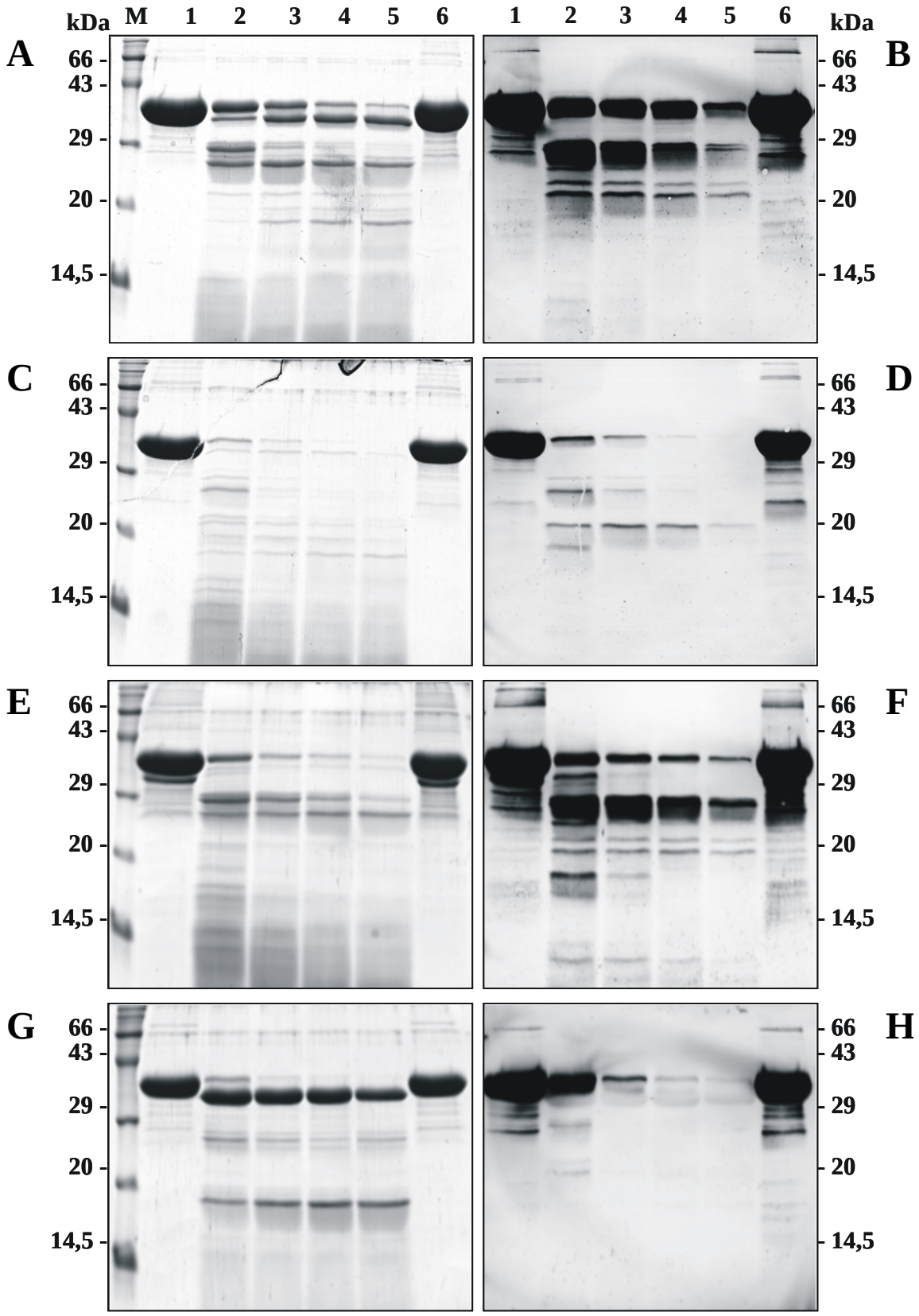

Fig. 5 Partial proteolysis by trypsin digestion. Coomassie-stained gels (A, C, E, G), Western blot using an antibody recognising the N-terminal 6x His tag (B, D, F, H). From the left to the right: M, protein standard; 1, before digestion; 2, 15 min; 3, 30 min; 4, 45 min; 5, 60 min digestion; 6, 60 min without trypsin. A, B AtStr1. C, D AtStr1wlink. E, F AtStr1C339V. G, H AtStr1C332S. Representative results out of three set of experiments are shown.

The mature AtStr1 protein has a predicted molecular mass of 35.5 kDa excluding the His_6-tag. Within the first 15 min about 50% of the protein is digested to smaller fragments of around 32, 29 and 27 kDa (Fig. 5A and 5B). As seen in the Coomassie-stained gel the amount of the 32 kDa fragment continuously increases (Fig. 5A). This fragment does not contain the His_6-tag as seen in the immuno blot analysis (Fig. 5B). Western blot analysis reveals that the 29 kDa is the most dominant band at the beginning of the proteolysis but it decreases rapidly within 60 min incubation to almost undetectable amounts. Part of the mature protein remains undigested even after 60 min of trypsin treatment. A small percentage of all four protein preparations seems to dimerise as visible in the Western blot analysis (Fig. 5B, D, F, H). Immediately after addition of trypsin the homodimers disappear.

The AtStr1wlink has a predicted molecular mass of 33.3 kDa. The difference in size is well visible in the SDS-PAGE analysis (Fig. 5C and 5D). The AtStr1wlink protein is proteolysed by trypsin to an uncountable number of smaller protein fragments within 15 min of digestion. It seems highly prone to degradation by a protease in agreement with results obtained previously (Burow et al., 2002). Already after 45 min none of the intact protein can be detected even by the very sensitive Western blot analysis (Fig. 5D). The progress of the proteolysis of AtStr1C339V (Fig. 5E and 5F) is comparable to the results obtained for mature AtStr1 (Fig. 5A and 5B). The degradation seems to progress slightly faster than that of AtStr1. The AtStr1C332S protein is immediately digested to a band of about 32 kDa (Fig. 5G and 5H). This degradation product and a 17 kDa fragment are relatively stable till the end of the trypsin incubation. N-terminal fragments containing the His_6-tag are almost undetectable already after 45 min of trypsin treatment. In summary, the results indicate that the AtStr1wlink can be easily digested by the trypsin protease. This high susceptibility might explain the highly reduced enzyme activity (up to 50%). But even single point mutations lead to either slightly different proteolysis behaviour (AtStr1C339V) or to completely different fragments and increased proteolysis (AtStr1C332S).

Discussion

Results from fluorescence spectroscopy in the present study show that the AtStr1 protein is persulfurated to a high degree. There are slight variations in the persulfuration percentage of the protein in diverse preparations although the recombinant protein was always prepared and purified in the same way. The presence of different concentrations of thiols, such as Cys, glutathione or DTT, in the dialysis buffer or directly added in the measuring cuvette did not influence the persulfuration state of AtStr1. The addition of cyanide in 10-fold molar excess or even in equimolar amounts showed that the persulfide formation could be completely removed. After removal of cyanide by gelfiltration the protein could be persulfurated again in a concentration dependent manner by both substrates chosen, 3-MP and TS. Although the K_m for TS was lower than for 3-MP a higher V_{max} was observed for 3-MP (Papenbrock & Schmidt, 2000a; Burow et al., 2002). The data from the present study could not resolve this contradiction. It can be concluded that the catalytically active Cys in the AtStr1 protein cycles in the reaction between the sulfur-free and the sulfur-substituted form as was hypothesised. It would be very interesting to compare active and inactive single-domain Str (AtStr14, AtStr15, AtStr16, and AtStr18) with respect to their persulfuration states and persulfide formation by different sulfur donors.

The percentages of the secondary structure of all four AtStr1 proteins determined by far UV CD are almost equal. The CD spectrum itself differs only for the AtStr1wlink protein (Fig. 4) indicating a shifting in the order of secondary structure parts in this protein.

The near UV CD analysis is mainly based on the position of aromatic amino acids in their chiral environment. The maximum emission wavelength of a tryptophan reflects the polarity of its environment. In the non-polar core of a globular protein the maximum emission wavelength is around 330 nm whereas in a hydrophilic environment the maximum emission wavelength reaches 355 nm. Thus any change in the maximum emission wavelength reveals a conformational change in a protein (Buhot et al., 2004). Interestingly, five out of the six tryptophan residues in the N-terminus of AtStr1 are highly conserved in all eukaryotic sequences (Papenbrock & Schmidt, 2000b). Therefore one can assume that their position in the tertiary structure is also highly conserved. This fact makes the near UV CD technique paticularly suitable for the analysis of conformational changes in mutagenised proteins.

Mainly for the AtStr1wlink protein differences in the shape of the CD spectrum could be observed. This protein produced the lowest signals in near-UV CD analysis (Fig. 3).

This indicates a more dynamic and flexible and consequently less stable tertiary structure of the protein. The removal of the plant specific linker-part led to a reduction in the enzyme activity to about 50% for 3-MP and 30% for TS (Burow et al., 2002). Obviously, the linker sequence plays an important role not only in providing a hydrophobic environment for substrate binding and catalysis as was shown previously (Finazzi Agro et al., 1972; Miller-Martini et al., 1994a, 1994b) but is also highly responsible for the generation of an ordered tertiary structure of the AtStr1 protein. Depleted of the linker elongation the protein loses a tight packing of the tertiary structure and thus it adopts the structural features of the so-called 'molten globule' state. It is noteworthy that although no tight tertiary structure is detected, the percentage of secondary structure of the protein often remains native-like (Buhot et al., 2004) like in the case of AtStr1wlink.

The linker in the AtStr1 peptide seems to play a different role than the shorter linker sequences usually found in two-domain eukaryotic Str. The alignment of several two-domain eukaryotic Str visualises the exceptional role of elongated plant linkers (Papenbrock & Schmidt, 2000b). The two-domain Str proteins from plants are localised in the cytoplasm (AtStr2) and in the mitochondria (AtStr1) (Bauer et al., 2004). The exact subcellular localisation of AtStr1 within the mitochondria is not yet known. To our knowledge there are no other mitochondrial proteins containing elongated linker sequences connecting two domains. Therefore comparisons with other proteins are not possible. The pH in the mitochondria is about pH 7.5 which is much higher than the predicted pI of AtStr1 (pI 5.1). In our studies a pH close to the mitochondrial pH was chosen to imitate the *in vivo* conditions. The deletion of the linker sequence and the point mutations of the Cys residues have negligible influences on the pI of the four recombinant proteins. The reduction of the pH to pH 5.0 changed the spectroscopic results only slightly.

Remarkably, the AtStr1C332S shows the highest signals in near UV CD analysis demonstrating that the rigidity of the molecule is high in agreement with results from proteolysis (Fig. 5G). The increased rigidity associated with the lack of the catalytic residue C332 could be due to the loss of distinct conformational states revealed in other Str, e.g. RhdA from *A. vinelandii* (Bordo et al., 2000).

AtStr1C339V has a slightly modified tertiary structure relative to the other proteins. The signals are flat especially in the tyrosine-tryptophan region of the spectrum. The signal might be low because in the region of 280 to 300 nm the signal can not be well resolved indicating the co-presence of multiple conformations due to an absence of rigidity, high flexibility or instability of the structure (Fig. 3).

For a final conclusion of the impact of conformational changes on catalysis the three-dimensional structure of the wild-type and mutated proteins need to be determined experimentally by X-ray analysis of protein crystals in the presence of substrates or analogues. So far only the three-dimensional structure of the non-catalytic Rhd homology domain of AtStr4 (At4g01050) has been resolved (Pantoja-Uceda et al., 2005).

In the proteolysis studies an antibody directed against the His_6-tag was used for Western blot analysis because this antibody recognised all four recombinant proteins with comparable sensitivity. The polyclonal antibody directed against the recombinant AtStr1 protein (Papenbrock & Schmidt, 2000a) showed a weaker reaction with AtStr1wlink than with the other three proteins (data not shown). One disadvantage of the His_6-tag antibody might be that it only recognises proteins with an intact N-terminus. On the other hand one can draw conclusions about different susceptibilities of either the N-terminus or the C-terminus with this antibody. In all four proteins analysed the N-terminus shows a higher accessibility for trypsin digestion than the C-terminus. Also the two domains of *A. vinelandii* Rhd show different resistance to endoproteinases, however, in this case the C-terminal is more prone to digestion than the N-terminal domain over the time (Melino et al., 2004).

So far, no data concerning protein stability of AtStr1C332S could be observed. The methods used in previous studies based on the determination of enzyme activity showing that the replacement of the catalytically active Cys C332 led to a complete loss of activity (Burow et al., 2002). Therefore the method of partial tryptic digestion can give new insights into the role of C332 on the overall stability of AtStr1. The first degradation product of AtStr1C332S is quite stable, and the overall stability and rigidity seems to be higher than that of the wild-type protein (Fig. 5A and G). This result is in agreement with the near UV CD analysis in which the strongest signals are found for AtStr1C332S indicating a high rigidity of the molecule.

The tryptic digestion pattern of AtStr1C3339V shows high similarity to that of AtStr1 indicating a similar accessibility of digestion sites and of comparable conformation in agreement with results obtained by spectroscopy. In a previous study the mutation of all nonessential Cys residues in bovine Rhd to serines turned the enzyme into a form (C3S) that was fully active but less stable than wild-type. In 1,1'-bis(4-anilino)naphthalene-5,5'-disulfonic acid (bis-ANS) binding studies it has been shown that in C3S more hydrophobic regions were exposed at the surface of the protein than in wild-type, although both had similar secondary structures suggesting the flexibility of its structure. bis-ANS bound to

C3S in its C-terminal domain as was shown by gel electrophoresis and proteolysis, and the binding made the C-terminal domain more susceptible to trypsin cleavage (Kaur et al., 2004). Although Rhd proteins display high sequence homologies to each other, they differ significantly in their physico-biochemical behaviour. Detailed experimental investigations are necessary individually for each protein.

The AtStr1wlink shows a very high susceptibility to trypsin. By the removal of the linker sequence elongation both domains probably are not able to adopt the correct positioning to each other. Therefore tryptic digestion sites usually buried inside the protein are more accessible to the endoprotease than in the wild-type protein. AtStr1wlink gave the lowest signals in near UV CD analysis. Both independent results indicate a high flexibility of the tertiary structure although the overall percentage of the secondary structures is almost unchanged. As consequences of site-directed mutagenesis in the linker sequence of bovine Rhd the protein was virtually indistinguishable from the wild-type protein in all properties tested, except for an increased susceptibility to perturbation compared to the wild-type protein (Luo et al., 1995). The elongation of the linker during evolution of the plant protein was obviously essential to provide full enzyme activity for plant specific substrates.

The complete loss of enzyme activity in AtStr1C332S as determined previously (Burow et al., 2002) can be explained by the missing catalytically active Cys but is also caused by conformational changes in the protein structure. The AtStr1C339V protein shows almost no differences in the overall secondary and tertiary structure, therefore the reduced enzyme activity (Burow et al., 2002) indicates a direct involvement of C339 in the catalytic mechanism. The reduced activity of AtStr1wlink is clearly based on higher flexibility and reduced stability of the protein molecule indicating the importance of the elongated linker sequence for the correct conformation of the protein.

CHAPTER III

Characterisation of group IV *Arabidopsis* sulfurtransferases

Abstract

Sulfurtransferases (Str) comprise a group of enzymes widely distributed in archaea, eubacteria, and eukarya which catalyse the transfer of a sulfur atom from suitable sulfur donors to nucleophilic sulfur acceptors. Since the identification of the *in vivo* substrates of the proteins has proven elusive thus far for all organisms investigated, specific biological roles for most members of this multi protein family have not been established. In the *Arabidopsis thaliana* genome 20 putative Str (AtStr) were identified and classified into six groups according to amino acid sequence homologies. The present work focuses on the characterisation of the three proteins AtStr9, AtStr10 and AtStr11 of group IV. All three proteins are single-domain proteins, containing one rhodanese (Rhd)-domain. The proteins display 39 to 59% similarity among each other on a local level. While AtStr9 with a size of 25.5 kDa and the 23.8 kDa protein AtStr10 appear to be active Str proteins, containing the catalytically important cysteine (Cys) residue in the active site loop, the 28.7 kDa protein AtStr11 lacks the catalytic Cys and thus its Rhd-domain must be considered inactive. There are no putative functions described for any of the three proteins. All three proteins have been expressed heterologously in *Escherichia coli*. However, the majority of the proteins was found in an insoluble state. Trials to express the proteins in a soluble form did not lead to satisfying amounts of soluble protein for the conduction of activity assays thus far. To determine the subcellular localisation of AtStr9, AtStr10 and AtStr11, fusion constructs of the proteins with the green fluorescent protein were transiently transformed into *Arabidopsis* protoplasts. The results indicate a localisation of AtStr9 in the chloroplasts, while AtStr10 seems to be localised in the mitochondria. For protein AtStr11 there are hints for a possible dual-localisation of the protein in chloroplasts and peroxisomes. However, the localisation studies done in this work do not suffice to unambiguously determine the subcellular localisation of the proteins and can only give hints for a possible localisation. The knowledge of the kind of substrate preferably used by the proteins *in vitro* and the subcellular localisation of the members of this multi protein family will be helpful for the elucidation of their specific functions in the organism.

Introduction

The sulfurtransferase (Str) / rhodanese (Rhd) multi protein family is very heterogeneous. The characteristic feature unifying all members of this multi protein family is the structurally well-defined Rhd homology domain. Rhd domains are ubiquitous structural modules that can be found as tandem repeats, with the C-terminal domain hosting the active-site cysteine (Cys) residue, as single domain proteins or as members of multi domain proteins (Bordo & Bork, 2002). Recent results show that Rhd domains are structurally related to the Cdc25 phosphatase catalytic domain and may share a common evolutionary origin (Bordo & Bork, 2002; Forlani et al., 2003; Hofmann et al., 1998). In *Arabidopsis thaliana* 20 proteins containing one or more Rhd-domains and Str-patterns were identified. They have been classified into six groups according to their amino acid sequence homology (see Table 1 Chapter I). Members of groups I and VI have been characterised in more detail in previous studies. Group I consists of two two-domain Str proteins (Papenbrock & Schmidt, 2000a,b), group VI consists of five proteins that contain only the C-terminal Str-pattern and thus possess similarity to the single-domain Str from bacteria (Bauer & Papenbrock, 2002). The three single-domain Str AtStr9, AtStr10 and AtStr11 in group IV have not been characterised so far and are the subject of this work.

Str proteins catalyse the transfer of a sulfur atom from a suitable sulfur donor to a nucleophilic sulfur acceptor. The most studied and best characterised Str is bovine Rhd (thiosulfate:cyanide Str, EC 2.8.1.1) which catalyses, *in vitro*, the transfer of a sulfane sulfur atom from thiosulfate to cyanide, leading to the formation of sulfite and thiocyanate (Westley, 1973). In *Arabidopsis* the two two-domain Str proteins of group I prefer 3-mercaptopyruvate (3-MP) over thiosulfate as sulfur donor *in vitro* (Papenbrock & Schmidt, 2000a,b), while two of the single-domain proteins in group VI have been shown to prefer thiosulfate over 3-MP as sulfur donor *in vitro* (Bauer & Papenbrock, 2002). But although *in vitro* assays show Str or phosphatase activity associated with Rhd or Rhd-like domains, specific biological roles for most members of this multi protein family have not been established (Spallarossa et al., 2001). Proposed functions of Str proteins include the detoxification of cyanide (Vennesland et al., 1982) and reactive oxygen species (Nandi et al., 2000), an involvement in sulfate assimilation (Donadio et al., 1990) and in the biosynthesis of thiamin or thiouridine (Lauhon & Kambampati, 2000; Palenchar et al., 2000) as well as iron-sulfur cluster biosynthesis or repair (Bonomi et al., 1977). Str might also activate other proteins by direct transfer of sulfane sulfur. Single Rhd domain proteins have been associated with specific stress conditions (Bordo & Bork, 2002). Furthermore,

the frequently observed association of catalytically inactive Rhd domains with other protein domains suggests a role in regulation and signalling for these inactive modules (Bordo & Bork, 2002). In plants a role in the mobilisation of sulfur for transport processes from older leaves to growing tissues has been suggested (Papenbrock & Schmidt, 2000b). In *Cucurbita maxima* (pumpkin) a Rhd-like protein showing similarities to AtStr17 was found in phloem exudates and is suggested to be involved in stress and defence reactions (Walz et al., 2004). Furthermore, a homologue of AtStr15 in tobacco, *Ntdin*, a senescence-associated gene, was described to be involved in molybdenum cofactor biosynthesis (Yang et al., 2003). And very AtStr13 was found to be involved in auxin-signalling (Zhao et al., 2003).

The high abundance of putative Str proteins in *Arabidopsis* as well as the wide variability in the amino acid composition of the active-site loop which plays a key role in substrate recognition and catalytic activity (Bordo & Bork, 2002; Forlani et al., 2003), suggest an involvement of the 20 AtStr proteins in distinct biological functions.

Str have been identified in different compartments in living organisms. In *Escherichia coli* eight Str proteins were identified; GlpE, a single-domain Str, was identified as cytoplasmic protein, while at least one two-domain Str was found in the periplasm (Ray et al., 2000). In the cyanobacterium *Synechococcus* sp. strain PCC 7942 a Rhd-like protein was localised in the periplasmic space and was suggested to be involved in the transport of specific sulfur compounds (Laudenbach et al., 1991). In mammalia two distinct Str proteins have been identified displaying 3-MP and thiosulfate specific activity, respectively. The 3-MP Str protein was localised in the cytoplasm and the mitochondria by immunogold-labelling and Western blot analysis, while the thiosulfate Str was exclusively detected in mitochondria, mainly in liver cells. 3-MP Str might detoxify cyanide in the cytoplasm. In the mitochondrion both Str enzymes may effectively protect cytochrome c oxidase (Nagahara et al., 1995, 1999). *Arabidopsis* Str proteins of groups I, II and VI have been located in the chloroplast, the mitochondrion and the cytoplasm by different methods (Hatzfeld & Saito, 2000; Heazlewood et al., 2004; Nakamura et al., 2000; Papenbrock & Schmidt, 2000a; Bauer et al., 2004).

The aim of our research is the functional analysis of all members of the nuclear encoded Str multi protein family in *Arabidopsis*. The more information is available on the *Arabidopsis* Str proteins, the more can the experiments be specified to identify their *in vivo* function in the plant organism. The knowledge of their substrate preference *in vitro* and

their subcellular localisation is a good prerequisite for the determination of their metabolic role in the multicellular organism.

Material & methods

Plant material

Seeds of *Arabidopsis thaliana* (L.) Heynh., ecotype C24, were originally obtained from the Arabidopsis Biological Resource Center, ABRC. Seeds were germinated on substrate TKS1 (Floragard, Oldenburg, Germany). Seedlings 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 µmol x m^{-2} x s^{-1} (sodium vapor lamps, SON-T Agro 400; Philips, Hamburg, Germany).

DNA cloning techniques

For the amplification of a cDNA sequence encoding AtStr9 (At2g42220), the full length EST clone RAFL07-16-M19 was obtained from RIKEN BioResource Center, Tsukuba, Japan. For the amplification of *AtStr10* (At3g08920) and *AtStr11* (At4g24750), a λYES cDNA library (Elledge et al., 1991) was used as template. The primer pairs used for the production of the expression clones and the construction of fusion clones with the green fluorescent protein (GFP) are listed in Table 2.

The PCR 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 about 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. For the construction of expression clones 28 PCR cycles of 60 s at 94°C, 60 s at 57°C for *AtStr9* and 56°C for *AtStr10* and *AtStr11*, and 60 s at 72°C were run. The process was finished with an elongation phase of 300 s at 72°C. The PCR products were ligated into the pQE-30 expression vector (Qiagen, Hilden, Germany) as described before (Papenbrock & Schmidt, 2000a). For the construction of GFP fusion clones 28 PCR cycles of 45 s at 94°C, 45 s at 55°C, and 45 s at 72°C each were run. The process was finished with an elongation phase of 420 s at 72°C. The amplified PCR fragments were ligated into pBSK-based enhanced GFP containing vectors as described previously (Bauer et al., 2004).

The pFF19GFP–SKL clone used as peroxisomal control in the localisation studies was kindly provided by A. Wachter, Heidelberger Institut für Pflanzenwissenschaften, Heidelberg, Germany (Nowak et al., 2004).

Table 1 Overview of the expression clones and GFP fusion clones produced. The name of the protein, the length of the PCR amplified DNA fragment, the primer pairs designed with the respective restriction sites and the respective vectors used are listed. More information on the respective members of the multi protein family are available at the website http://arabidopsis.org/info/genefamily/STR_genefamily.html. GFP, green fluorescent protein; no., number.

Protein name	Base no.	Primer pairs	Restriction sites	Vector
AtStr9	700	P124 5'-gga tcc gcg ggg atc ata agc cct a-3'	*Bam*HI	pQE-30
		P125 5'-gtc gac gct tgt tgg agg aaa gag c-3'	*Sal*I	pQE-30
	702	P170 5'-cca tgg cgg gga tca taa gcc cta-3'	*Nco*I	pGFP-N
		P171 5'-gga tcc gct tgt tgg agg aaa ga-3'	*Bgl*II	pGFP-C
AtStr10	642	P120 5'-gga tcc aca gtt ctt ctt cct cag-3'	*Bam*HI	pQE-30
		P121 5'-ctg cag acg act ctt ctg gcc aaa a-3'	*Pst*I	pQE-30
	642	P140 5'-cca tgg cag ttc ttc ttc ctc-3'	*Nco*I	pGFP-N
		P168 5'-aga tct acg act ctt ctg gcc aa-3'	*Bgl*II	pGFP-C
AtStr11	780	P126 5'-gga tcc gag tct cta tct ctt ccc-3'	*Bam*HI	pQE-30
		P127 5'-ctg cag atg gcc tct taa ctc ctg a-3'	*Pst*I	pQE-30
	780	P144 5'-cca tgg agt ctc tat ctc ttc cc-3'	*Nco*I	pGFP-N
		P169 5'-aga tct atg gcc tct taa ctc ctg-3'	*Bgl*II	pGFP-C

Expression, extraction and purification of AtStr proteins in *E. coli*

For the heterologous expression of the *Arabidopsis* Str-like proteins the respective expression vectors were transformed into the *E. coli* strains XL1-blue and BL21. The recombinant proteins were expressed under different conditions: after growth of the respective *E. coli* cultures at 37°C to an OD_{600} of 0.6 in Luria Bertani (LB) medium (10 g tryptone, 5 g yeast extract and 10 g NaCl per litre; pH 7.0), 2x yeast-tryptone (2YT) medium (16 g tryptone, 10 g yeast extract and 5 g NaCl per litre; pH 7.0) or Terrific Broth

(TB) medium (2.3 g KH_2PO_4, 16.4 g K_2HPO_4, 10.8 g tryptone, 21.6 g yeast extract and 3.6 ml glycerol per litre) containing ampicillin (100 µg x l^{-1}) (AppliChem, Darmstadt, Germany), expression was carried out for 1 to 3 h at 37°C after induction with 0.5 mM and 1 mM final concentration of isopropyl-β-D-galactoside (IPTG; AppliChem). To avoid the formation of inclusion bodies the expression of the proteins was alternatively carried out at 30°C for 3 h. Cell lysis was obtained by adding lysozyme (final concentration 1 mg x ml^{-1}; Roth) and vigorous homogenising. The recombinant proteins were purified under non-denaturing conditions by affinity chromatography with the nickel-affinity resin according to the manufacturer's instructions (Qiagen). The eluted fractions were dialysed overnight with excessive amounts of 20 mM Tris/HCl, pH 7.5 at 4°C, concentrated and the protein concentration was estimated.

To extract protein AtStr9 from the insoluble fraction, pellets of AtStr9 expression cultures were lysed and centrifuged after the standard purification protocol (Qiagen) and then treated with sonication, detergents or a combination of both. (a) An aliquot of the pellet was solved in 2 ml 1% [v/v] CHAPS in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and incubated for 1 h on ice at 60 rpm on a shaker. The solution was centrifuged for 10 min at 10,000*g* and 4°C. (b) An Aliquot of the pellet was sonicated (40 W) for 5 min, then centrifuged 20 min at 10,000*g*, 4°C. (c) An Aliquot of the pellet was sonicated (40 W) for 10 s and afterwards incubated on ice for 10 s. This was repeated six times followed by four times washing for 5 min with a solution containing 0.25% [v/v] Tween 20 and 0.1 mM EGTA and centrifugation for 5 min at 10,000*g*, 4°C after each washing. Aliquots of pellets and supernatants were mixed with sample buffer and denatured for 15 min at 95°C.

Expression, purification and extraction of the proteins were checked by SDS-PAGE and Western blot analysis.

SDS-PAGE and Western blot analysis

Denaturing gel electrophoresis of protein samples was done according to Laemmli (1970). Samples were heated at 95°C in sample buffer (56 mM Na_2CO_3, 56 mM dithiothreitol, 2% [w/v] SDS, 12% [w/v] sucrose, 2 mM EDTA) for 15 min. Cell debris was removed by centrifugation and supernatants were directly loaded onto the SDS gel. The resulting gels were either stained with Coomassie Brilliant Blue to visualise the proteins or blotted onto nitrocellulose membranes (Sambrook et al., 1989). For immunodetection RGS anti-His antibody (Qiagen), anti-mouse Ig (Sigma) and anti-rabbit antibody conjugated with

alkaline phosphatase (Sigma) were used successional. Colorimetric detection was done using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as substrates for alkaline phosphatase.

Transient expression of GFP fusion constructs in *Arabidopsis* protoplasts and microscopic analysis

Polyethylene glycol transmitted transformation of *Arabidopsis* protoplasts with GFP fusion constructs was performed as described in Bauer et al. (2004). The transiently transformed protoplasts were analysed with an Axioskop microscope (Carl Zeiss, Jena, Germany). The GFP fluorescence was collected with a band pass filter (BP 450-490) for excitation and with a long pass filter (LP 520) for emission. The generation of bright field transmission micrographs for visualisation of non-fluorescent protoplast structures was achieved using the manufacturer´s filter settings. All images were edited with Corel Photo Paint 10. The transformations were performed with each clone at least five times resulting always in the same subcellular localisation.

Miscellaneous

Protein estimation was carried out according to Bradford (1976) using bovine serum albumin as protein standard. The analyses of DNA and amino acid sequences were performed with the programs EditSeq and MapDraw in DNASTAR (Madison, WI, USA) and ClustalW and t-Coffee (http://www.ebi.ac.uk/tools; Notredame et al., 2000). For the identification of protein domains SMART was used (http://smart.embl-heidelberg.de). For the computer-based prediction of the protein localisation different programs were applied (mainly PSORT, TargetP, Predotar, TMHMM and further programs in http://www.expasy.ch/tools).

Results

Identification and *in silico* characterisation of the putative *Arabidopsis* sulfurtransferase proteins of group IV

On the characterisation of the three proteins of group IV: All three proteins, AtStr9, AtStr10 and AtStr11, are single-domain proteins. Neither of them has been characterised so far nor does either of them show any similarity to known proteins. In the databases they are annotated as ‘hypothetical’, ‘unknown’, ‘putative’ or simply as ‘rhodanese-like family proteins’ (Table 3). The group is quite heterogeneous displaying 39.0 to 58.5% similarity

among each other on a local level. The similarity to the two-domain AtStr1 is 34.5%, 53.7% and 31.2% on a local level for the respective proteins. The 25.5 kDa protein AtStr9 and the 23.8 kDa protein AtStr10 seem to be active Str proteins, containing the catalytically important Cys residue in the active site loop (C145 and C142, respectively), while in AtStr11 (28.7 kDa) the catalytic Cys is missing and thus its Rhd-domain must be accounted inactive. AtStr10 and AtStr11 each contain one Cys residue in the amino acid sequence, while AtStr9 contains three Cys residues in total (C85, C145 and C168). None of the three proteins shares the active-site motifs of 3-MP Str CG(S/T)GVTA or eukaryotic thiosulfate Str and Rhd CRKGVTA as given in Williams et al. (2003) (Fig. 1).

```
AtStr11         MESLSLPVLNPLLASGSNL-------FRNQHSRMTSSMVSS-------------------
AtStr10         M-----TVLLPQLNHIHKL-----------------------------------------
AtStr9          MA----GIISPSPTALYFT-----------------------------------------
AtStr1          M-----ASTGVETKAGYSTSSVSTSEPVVSVDWLHANLREPDLKILDASWYMPDEQRNPI
                *

AtStr11         -------------L-------KSPIGGTSLSTVRRFGVGVVRMQAVDED-IDLKQMRDIA
AtStr10         ----------------------PVVLNRRLRQ-SYRLPVI--------S-AVSGKE----
AtStr9          ----------------------SNVGGRRLKAVSWAGKSV------SGN-VIRRRS----
AtStr1          QDYQVAHIPRALFFDLDGISDRKTTFAHMLPTEEAFAAGCSALGIDNKDEVVVYDGKGIF
                                       *                    .

AtStr11         AAKKRWDGLLREGKVK-------LLTPREAGYA-ISLSNKPLLDVRPSSERNKAWIKGST
AtStr10         --------LILSGKVR-------AVEPKEANAV-VASEGYILLDVRPAWEREKARVKGSL
AtStr9          --------LRIAAELK-------FVNAEEAKQL-IAEEGYSVVDVRDKTQFERAHIKSCS
AtStr1          SAARVWWMFRVFGHEKVWVLDGGLPRWRASGYVESSASGDAILKASAASEAIEKIYQGQT
                        :   .. :           . :     : ..  ::..    :  .   :.

AtStr11         WVPIF-DNDDNLDAGTLSKKVTSFAMGGWWSGAP------TLSFNRYV-C----------
AtStr10         HVPLF-VEDPDNGPITLLKKWIHLGYIGLWTGQR------FTMINDEFALRVV-------
AtStr9          HIPLF-IYNEDNDIGTIIKRTVHNNFSGLFFGLP------FTKVNPEF-LKSV-------
AtStr1          VSPISFQTKFQPHLVWTLDQVKNNMEDPTYQHIDARSKARFDGTAPEP-RKGIRSGHIPG
                  *:    . :       .:         :

AtStr11         ------------------------------------------------------ERYE
AtStr10         --------------------------EAVPDKESKVLVVCGEGLRSLAAVSKLHGEGYK
AtStr9          --------------------------RNEFSQDSKLLLVCQEGLRSAAAASRLEEAGYE
AtStr1          SKCIPFPQMFDSCNTLLPAEELKKRFDQEDISLDKPIMASCGTGVTACILAMGLHRLGKT

AtStr11         NLFWVQGGLESAQDEDLVT-EGVQPLKLAGIGGFSEFLGWTDQQRAQAAKEGWGYRLVYT
AtStr10         SLGWLTGGFNRVSEGDFPEIEGTEELRFATIGGVSFYL---------------------
AtStr9          NIACVTSGLQSVKPGTFESV-GSTELQNAGKAGLITIQ------------------GKI
AtStr1          DVPIYDGSWTEWATQPDLPIESVE-----------------------------------
                .:    ..             .

AtStr11         ARLFGVVLAADALFVGAQQLGHYIQELRG--H
AtStr10         -----------LKLL--VLLPSFGQKSR----
AtStr9          SAVLGTVLVCAYLFI--QFFPDQAEKLFPPTS
AtStr1          ----------------------------SSS
```

Fig. 1 Alignment of group IV single-domain Str sequences from *Arabidopsis*. The sequence of the two-domain protein AtStr1 (CAB53639) and the full-length protein sequences of AtStr9 (AAB88647), AtStr10 (AAF07833) and AtStr11 (CAA22988) were aligned using the global alignment algorithm in t-Coffee. Identical amino acid residues are marked with an asterisk, conserved substitutions with a colon and semi-conserved substitutions with a single dot. The active site cysteine residue is underlined and the Rhd domains according to SMART are shaded grey.

For the computer based prediction of the subcellular localisation several computer programs using different algorithms were applied. The results of the prediction by computer programs are summarised in Table 2. According to these data, AtStr9 will be localised most probably in the chloroplasts, AtStr10 in the mitochondria or the plasma membrane and AtStr11 in the chloroplasts or the peroxisomes. Predotar and TargetP give agreeing results for all three proteins while the predictions of the PSORT program differ from the other programs. Furthermore computer programs predicting transmembrane domains (e.g. TMHMM, PSORT; data not shown) were applied. According to those data none of the proteins may have a domain fully traversing a membrane. However, AtStr9 and AtStr10 may be anchored in a membrane with the C- terminus, while AtStr11 is predicted to be soluble.

Table 2 *In silico* characterisation of sulfurtransferases AtStr9, AtStr10 and Atstr11 from *Arabidopsis.* The protein name, gene identification, protein description, and the number of amino acids and MW are summarised. The programs Predotar, PSORT, and TargetP were used for the localisation prediction (http://www.expasy.ch/tools). The respective probabilities are given. Abbreviations: aa, amino acids; ass., associated; c, chloroplast; er, endoplasmic reticulum; ID, identification; kDa, kilo Dalton; m, mitochondrial; MW, molecular mass; no., number; SP, signal peptide; tm, transmembrane; TP, targeting peptide.

Protein name	Gene ID	Description	no. aa	MW (kDa)	Localisation prediction		
					Predotar	PSORT	TargetP
AtStr9 AAB88647	At2g42220	hypothetical protein	234	25.5	chloroplast -0.992 mitochondrion -0.007	er (membrane) – 0.850 chloroplast thylakoid membrane – 0.484 plasma membrane – 0.440 microbody (peroxisome) – 0.395	cTP - 0.913 mTP – 0.126 SP – 0.020
AtStr10 AAF07833	At3g08920	unknown protein	214	23.8	mitochondrion - 0.996 chloroplast - 0.000	plasma membrane – 0.700 microbody (peroxisome) – 0.500 er (membrane) – 0.200 chloroplast thylakoid membrane – 0.145	mTP – 0.590 cTP – 0.232 SP – 0.024
AtStr11 CAB22988	At4g24750	putative protein	260	28.7	chloroplast - 0.998 mitochondrion - 0.002	microbody (peroxisome) – 0.748 chloroplast stroma – 0.562 chloroplast thylakoid membrane – 0.343 chloroplast thylakoid space – 0.269	cTP - 0.911 mTP – 0.197 SP – 0.025

Expression and purification of recombinant *Arabidopsis* sulfurtransferases

For the heterologous expression of the *Arabidopsis* Str proteins AtStr9, AtStr10 and AtStr11 in *E. coli*, the respective cDNA sequences coding for the proteins, including the targeting peptide sequence, were cloned into the pQE-30 expression vector behind an N-terminal 6xHis-tag coding sequence and transformed into the *E. coli* strain XL1-Blue. All three proteins were successfully expressed in *E. coli*. AtStr9 (25.5 kDa) and AtStr10 (23.8 kDa) were expressed to a high extent, while the expression level of AtStr11 (28.7 kDa) remained at a lower level (Fig. 2 A). The size of the respective protein bands was verified via Western blot analysis of aliquots of the bacterial cultures after 2 h of expression using a monospecific RGS anti-His antibody. The heights of the protein bands on the membrane are bigger than the expected molecular masses of the proteins but have approximately the right size (Fig. 2C).

However, the majority of all three proteins was found in the insoluble fraction after extraction from the bacterial cells (Fig. 2B, lanes P and L). Trials to optimise the expression conditions with regard to a higher expression level of AtStr11 and higher solubility of all three proteins included modifications of incubation time and temperature, reduction of the concentration of IPTG used for induction of the T5-promotor and changing the culture volume. The best results for AtStr9 and AtStr10 concerning the expression level could be obtained in 50 to 200 ml LB culture induced with 1 mM IPTG and expression at an incubation temperature of 37°C for 2 h. For AtStr11 induction of 50 to 200 ml LB culture using 0.5 mM IPTG followed by expression at 30°C for 2 h gave best results. Trials to optimise expression by transformation of the proteins into *E. coli* strain BL21 also remained without improvement with regard to solubility of the proteins. Additional attempts to extract protein AtStr9, which gave the highest expression rate of the three proteins, from the insoluble fraction with detergents and sonication were partly successful but did not yield satisfying amounts of soluble protein. The best result was obtained by treatment of the pellet with low concentrations of detergents Tween 20 (0.25%) and EGTA (0.1 mM) in combination with repeated sonication (data not shown).

All three proteins were purified by standard nickel-affinity chromatography; elution fractions were combined, dialysed and concentrated. Because of the small amount of protein in the lysate (soluble fraction) from which the proteins were purified, the concentration of the proteins in the elution fractions was too low to give a band in the Coomassie-stained SDS-gel. Also in more sensitive Western blot analyses of purifications of the proteins there was no band detectable in the elution fractions (data not shown). The

~66 kDa band in the elution fraction of the purification of AtStr10 is an artefact with unknown origin. It is no dimer of AtStr10. This could be excluded by Western blot analysis. In this blot a single signal of approximately the size of AtStr10 was detected (Fig. 2C).

The concentration of all three proteins was not sufficient to cover the demands of the enzyme activity assays. The concentration of the purified and concentrated proteins remained below the detection level of the Bradford assay.

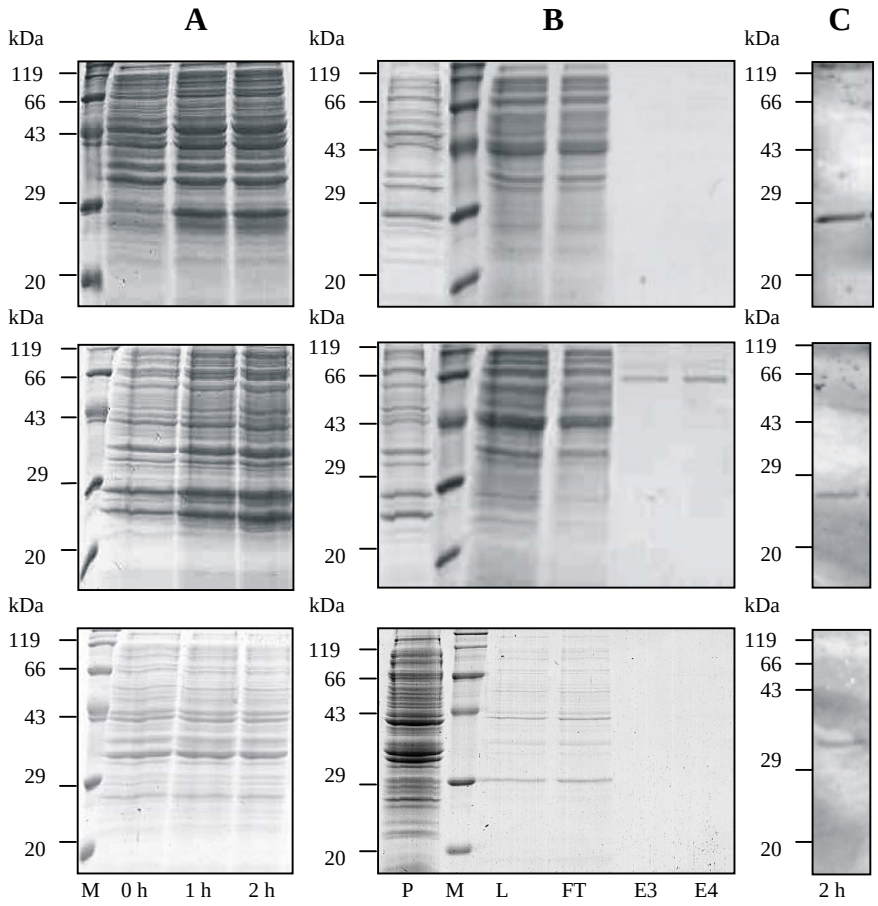

Fig. 2 Expression and purification of *Arabidopsis* Str AtStr9, AtStr10 and AtStr11 in *E.coli*. **A**, SDS-PAGE of expression in *E. coli*; **B**, SDS-PAGE of purification; **C**, Western blot of 2 h expression in *E. coli* (left to right). SDS-PAGE was performed according to Laemmli (1970). Aliquots were loaded on SDS gels. Western blot was performed using RGS anti-His antibody produced in rabbit and alkaline phosphatase coupled anti-rabbit antibody as secondary antibody. Lanes described from left to right: M, protein standard; 0 h, protein extract of transformed *E. coli* XL1-blue shortly before induction of the culture with isopropyl-β-D-galactoside; 1 h and 2 h, transformed *E. coli* XL1-blue protein extract 1 h and 2 h after induction; P, pellet after cell lysis and centrifugation; L, lysate after cell lysis and centrifugation; FT, flow through; E3 and E4 eluate fractions 3 and 4 from purification.

Subcellular localisation of AtStr9, AtStr10 and AtStr11

To determine the subcellular localisation of AtStr9, AtStr10 and AtStr11, N-terminal and C-terminal fusion constructs of the proteins and GFP were produced and transiently

transformed into *Arabidopsis* protoplasts. The *in vivo* subcellular localisation of the six GFP fusion constructs was visualised by fluorescence microscopy. The corresponding bright field picture visualises the protoplasts' cell membrane and the position of the chloroplasts and demonstrates the intactness of the protoplasts.

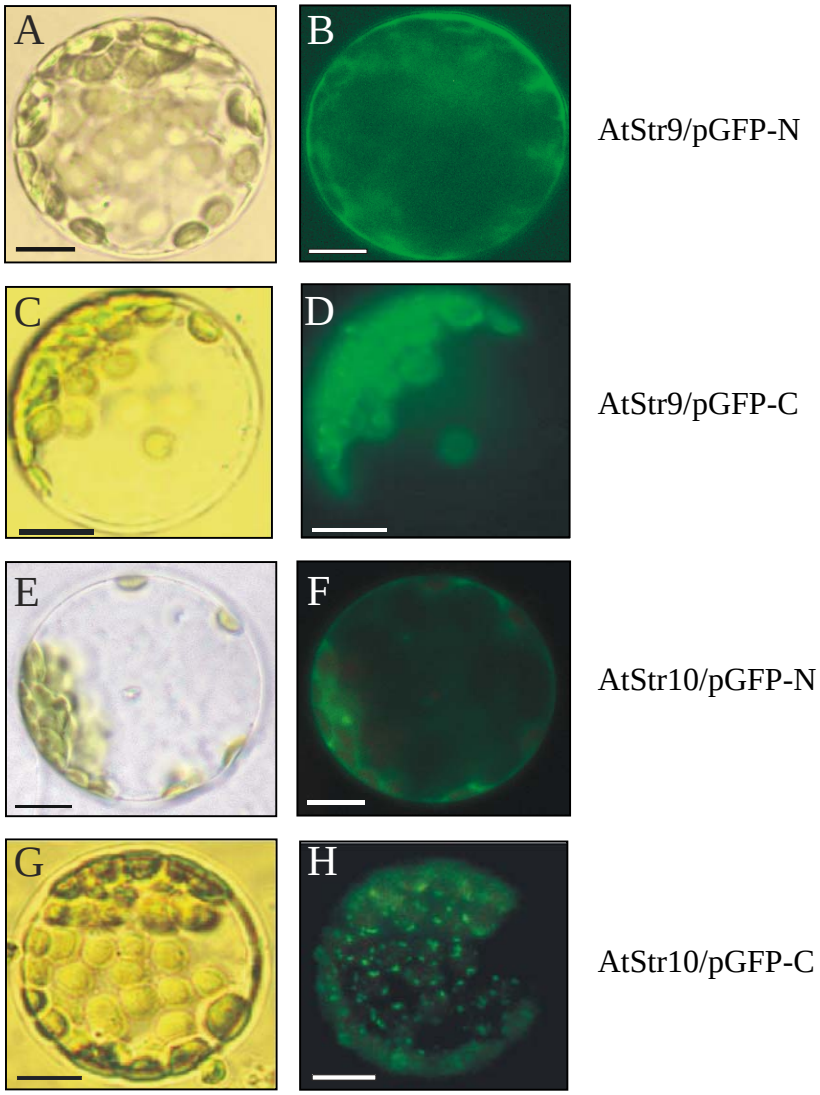

Fig. 3 Subcellular localisation of *Arabidopsis* sulfurtransferases AtStr9 and AtStr10. Fusion constructs of AtStr9 and AtStr10 with pGFP-N and pGFP-C were introduced into *Arabidopsis* protoplasts. The transiently transformed protoplasts were incubated overnight at room temperature and analysed using an Axioskop microscope with filter sets optimal for GFP fluorescence (BP 450-490/LP 520) (right). Bright field images (left) of the same protoplasts have been made to show cell membrane and chloroplast localisation.

The results indicate a localisation of AtStr9 in the chloroplasts (Fig. 3C, D). GFP fluorescence can be observed evenly distributed in the chloroplasts and in some brighter spots inside or in close vicinity to the chloroplasts. AtStr10 seems to be localised in mitochondria that are associated to the chloroplasts or inside or at the chloroplasts themselves (Fig. 3G, H). GFP fluorescence occurred as numerous small and bright spots close to, at the surface or even inside the chloroplasts. The fusion constructs of *AtStr9* and

AtStr10 with the GFP encoding cDNA sequence at the 5' end resulted in a fluorescence image identical to the transformed pGFP-C vector alone (data not shown) indicating that the constructs remained in the cytoplasm without being translocated (Fig. 3B, F).

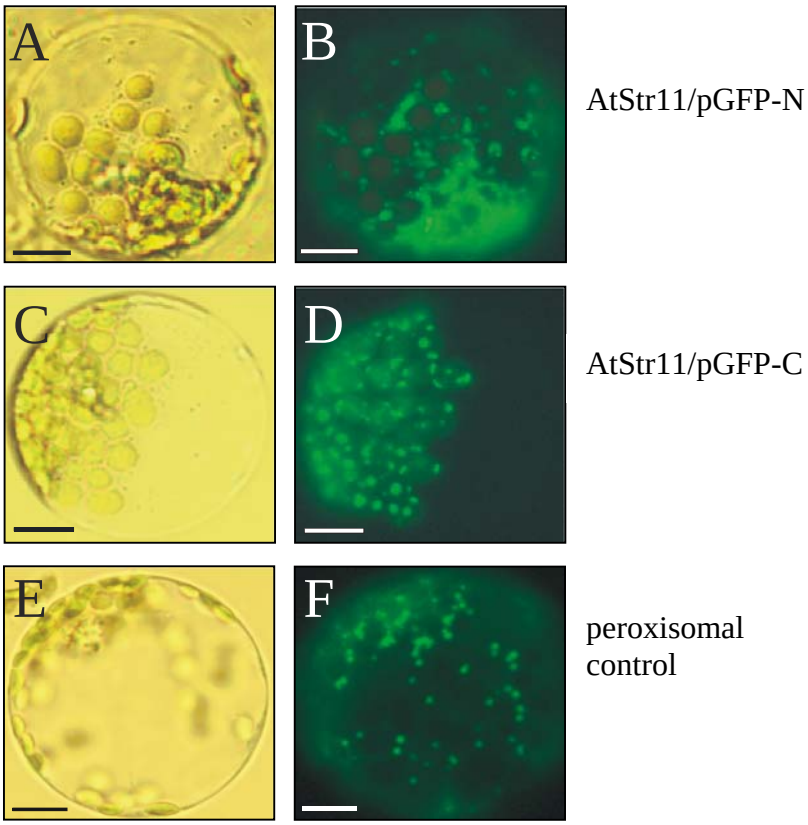

Fig. 4 Subcellular localisation of *Arabidopsis* sulfurtransferase AtStr11. Fusion constructs of AtStr11 with pGFP-N and pGFP-C and pFF19GFP-SKL as a control for peroxisomal localisation were introduced into *Arabidopsis* protoplasts. The transiently transformed protoplasts were incubated overnight at room temperature and analysed using an Axioskop microscope with filter sets optimal for GFP fluorescence (BP 450-490/LP 520) (right). Bright field images (left) of the same protoplasts have been made to show cell membrane and chloroplast localisation.

For protein AtStr11 both fusion constructs, AtStr11/pGFP-N and AtStr11/pGFP-C, show a specific distribution within the protoplasts: In the images of the pGFP-N construct many small fluorescing spots were observed. Here the chloroplasts were clearly not fluorescing (Fig. 4A, B). The image resembles that of the peroxisomal control construct given in Fig. 4F. When the pGFP-C construct was used fluorescence occurred evenly distributed all over the chloroplasts and also in bright spots inside or at the chloroplasts (Fig. 4C, D). These results indicate a possible dual-localisation of the protein in peroxisomes and chloroplasts.

Discussion

In the genome of *Arabidopsis* 20 putative Str proteins are encoded. In this work the three single domain proteins AtStr9, AtStr10 and AtStr11 of group IV have been characterised.

The proteins were expressed heterologously in *E. coli*. The purified proteins were expressed in an insoluble state and therefore could not be catalytically characterised in enzyme activity assays *in vitro*. In previous studies two two-domain AtStr proteins have been characterised showing a preference for 3-MP over thiosulfate as sulfur donor substrate (Papenbrock & Schmidt, 2000a,b) and two single-domain AtStr preferred thiosulfate over 3-MP *in vitro* (Bauer & Papenbrock, 2002). Both substrates used in these *in vitro* assays could also be metabolised *in vivo*. A leishmanial Str LmajMST (Williams et al., 2003), the *E. coli* GlpE protein (Ray et al., 2000) and bovine liver Rhd (Nandi & Westley, 1998) are able to use cyanide and thioredoxin as sulfur acceptor substrate *in vitro*. Recent investigations have shown that Rhd domains are also structurally related to the catalytic subunit of Cdc25 phosphatase enzymes and that the two enzyme families are likely to share a common evolutionary origin (Hofmann et al., 1998). The substrate specificity of the proteins is believed to be determined by the size and amino acid composition of the catalytic loop (Bordo & Bork, 2002). Biochemical data support the hypothesis that Rhd domains displaying a seven-amino-acid active-site loop are able to bind substrates containing phosphorus or the chemically similar arsenic, whereas Rhd-like domains displaying a six-amino-acid loop with Cys at the first position interact with substrates containing reactive sulfur or, in some cases, selenium (Ogasawara et al., 2001). However, the *in vivo* substrates of most members of the Str / Rhd multi protein family have not been identified. The putative AtStr proteins thus may use not only substrates containing sulfur but also substrates containing phosphorus, arsenic or selenium. Recently, AtStr5 has been shown to be a tyrosine phosphatase (Landrieu et al., 2004), while in first investigations on phosphatase activity in AtStr proteins no phosphatase activity could be determined for the six proteins analysed so far (Hartmann & Papenbrock, unpublished data).

However, in this study it was not possible to carry out these experiments with proteins AtStr9, AtStr10 and AtStr11 because the proteins could not be expressed in their soluble form and thus could not be purified in an amount big enough to cover the requirements of the enzyme activity assays. The majority of the expressed proteins was found in the insoluble fraction after the extraction from the bacterial cells. Data from computer based predictions of possible transmembrane regions in the sequences of the proteins indicate a

possible membrane association of the C-terminus of the sequence to a membrane in case of AtStr9 and AtStr10. This is supported by comparison of fluorescence micrographs of the thylakoid membrane protein AtStr15 (Bauer et al., 2004) with those of proteins AtStr9 and AtStr10. The images of AtStr9 and AtStr10 generated in this study display similarity to the images of the membrane protein concerning the distribution pattern of the fluorescent GFP constructs inside the cell. AtStr9 has also been found to be part of the proteome of the thylakoid membrane by three phase partitioning and mass spectrometry (Peltier et al., 2004). This would explain, why the proteins are found in the insoluble fraction after lysis of the bacterial cells. The proteins would pellet together with the associated membrane fragments during centrifugation. However, this does not explain, why also AtStr11 is expressed in an insoluble state. This protein is predicted to be soluble by all different programs applied. AtStr11 has additionally been expressed as a fusion construct together with thioredoxin in *E. coli* BL21 to produce the protein in a soluble state. After successful purification thioredoxin will be eliminated by proteolysis to obtain AtStr11 in a state in which enzyme activity can be determined. With this construct high expression levels of AtStr11 could be achieved. Still the majority of the expressed protein is found in the insoluble fraction. The heterologous overexpression of proteins in *E. coli* often results in the formation of inclusion bodies. The proteins accumulate in the cells as inactive, misfolded and insoluble aggregates. The proteins enclosed inside inclusion bodies may be purified under denaturing conditions using 6 M guanidine hydrochloride or 8 M urea added to the lysis buffer. As a consequence, the proteins must be properly refolded to regain activity. However, it is difficult and may be very time consuming to empirically define the right conditions that promote refolding of each single target protein into its native conformation to perform enzyme activity assays. And even if the right conditions for renaturing of the protein are found the recovery rates are often poor when a refolding step is included in the purification protocol. In our lab, trials to renature proteins that had been purified under denaturing conditions so far remained without success.

The membrane-associated proteins AtStr9 and AtStr10 may be purified after a protocol for membrane proteins. Peripheral membrane proteins that are only loosely bound to the membrane may be separated from the membrane fraction by increased pH, EDTA or low concentrations of non-ionic detergents like Tween or Triton (Lottspeich, 1998). Among the different extraction protocols applied in this work to extract the membrane-associated protein AtStr9 from the insoluble fraction, the treatment with Tween 20 and EGTA in combination with sonication gave the best results. Nevertheless, the amount of soluble

protein was not enough to gain sufficient amounts of protein from the purification for activity assays. Still, this may be a starting point for further trials to extract the proteins from the insoluble fraction to gain enough protein in the native state to characterise the proteins of group IV on the catalytic level. The question for activity and function of the three putative *Arabidopsis* Str proteins AtStr9, AtStr10 and AtStr11 thus remains a matter of debate.

While many members of the heterogeneous Rhd homology superfamily are known to be enzymatically active as Str or phosphatases the Rhd domain also occurs in the non-catalytic part of proteins in a catalytically inactive form lacking the catalytically important Cys residue. The Cys residue in the active centre of the protein is essential for Str activity because it covalently binds sulfur during the reaction. It appears likely that the proteins carrying a conserved Cys are functional as enzymes, most probably hydrolysing or transferring an anion via an intermediate covalently bound to the Cys residue during catalysis (Hofmann et al., 1998). In the inactive Rhd-domains the catalytic Cys is often replaced by acidic or non-polar residues. Apart from the N-terminal domain of the Rhd itself inactive Rhd homology domains could be identified in the non-catalytic regions of two distinct families of MAP-kinase phosphatases, in the non-catalytic domain of several ubiquitinating enzymes including yeast UBP4, UBP5, UBP7, human UBP8 and the murine deubiquitinating enzyme UbpY, in several phosphate and sulfate transferring enzymes and in a large and heterogeneous group of bacterial and eukaryotic stress response proteins (Fauman et al., 1998; Hofmann et al., 1998). The frequently observed association of catalytically inactive Rhd modules with other distinct protein domains suggests a regulatory role for these inactive domains, possibly in connection with signalling (Bordo & Bork, 2002). In catalytic investigations on the role of the non-catalytic N-terminal domain of the two-domain Str AtStr1 in *Arabidopsis* the C-terminal domain and the N-terminal domain have been expressed separately from each other. In activity assays it could be shown that the catalytic activity of the C-terminal domain hosting the catalytic Cys alone was boosted by addition of the N-terminal domain (Burow et al., 2002). AtStr11 lacks the catalytic Cys and thus probably also lacks activity. The Cys residue is replaced by a neutral alanine residue in this protein. The biological role of the inactive Rhd domains is still to be elucidated. Until now no protein consisting of only the inactive Rhd domain has been described. A possible function of the AtStr11 protein is questionable.

For the elucidation of the biological function of a protein the knowledge of its subcellular localisation might be of high value. Especially in plants the compartmentation

of the cell plays an important role in regulation and communication of cellular processes (Papenbrock & Grimm, 2001). *Arabidopsis* Str proteins have been located in the chloroplast (AtrStr14, AtStr15 thylakoid membrane, AtStr16), the mitochondrion (AtStr1, AtStr3) and the cytoplasm (AtStr2, AtStr18) by different methods (Hatzfeld & Saito, 2000; Heazlewood et al., 2004; Nakamura et al., 2000; Papenbrock & Schmidt, 2000a; Bauer et al., 2004). The results from the localisation studies on the proteins of group IV conducted in this work are more or less conform with the localisation prediction using several computer programs based on different algorithms. AtStr9 seems to be localised in the chloroplasts of the cells. This finding is in agreement with results from three phase partitioning and mass spectrometric analyses of the proteome of the chloroplasts thylakoid membrane. In these studies AtStr9 as well as AtStr4, AtStr4a and AtStr14 were located in the thylakoid membrane of the chloroplasts (Peltier et al., 2004). First preliminary immunomicroscopic analyses using a monospecific antibody against GFP also indicate a chloroplastic localisation of AtStr9 (personal communication, Prof. Dr. W. D. Sierralta, Laboratory of Ultrastructure, INTA University of Chile). Concerning the localisation of AtStr10 and AtStr11 the results obtained in this work are the only data available. The proteins have not been the subject of any other localisation study until now. Based on the fluorescence micrographs and computer-based predictions AtStr10 may be localised in mitochondria associated to the chloroplasts or in or at the chloroplasts themselves. AtStr11 may display a dual-localisation in chloroplasts and peroxisomes.

The localisation of the putative Str proteins of group IV can not finally be determined in this work. From fluorescence microscopic analyses of the distribution of GFP fusion constructs transiently transformed into *Arabidopsis* protoplasts we can get hints for a possible localisation of the proteins but the correct localisation cannot be concluded from these studies alone. Therefore, the subcellular distribution of the proteins will be analysed in further detail by transmission electron immunomicroscopy using a monospecific antibody against GFP. Additionally, Western blot analyses of organelle fractions isolated from *Arabidopsis* plant material (Fukao et al., 2002; Hausmann et al., 2003; Jensen & Bassham, 1966) using monospecific antibodies against the respective proteins will be done to confirm the proposed localisations derived from the present studies.

The future aims will be to elucidate the physiological sulfur donor(s) and acceptor(s) of the proteins and to estimate, whether and if which other proteins (e.g., thioredoxins) may act as sulfur donors for the formation of the persulfide at the active site of the proteins or as sulfur acceptor(s) to be able to pin the proteins onto specific metabolic pathways.

Furthermore, it will have to be elucidated, if the proteins may even act in the transfer of compounds other than sulfur (e.g. phosphorus, arsenic or selenium) or act in the regulation of specific reactions. The knowledge of the subcellular localisation of the members of this multi protein family will further give valuable indications for the elucidation of their specific functions in the organism. Recently, the expression level of a homologue of AtStr9 in the cadmium-tolerant *Datura innoxia* has been shown to be increased by cadmium in a suppressive subtractive hybridisation used to create a library enriched in cadmium-induced cDNAs. The coupling of a thiosulfate reductase to the Cys biosynthetic pathway by a Str may help to provide sulfide for Cys biosynthesis independent of the regular sulfate assimilatory pathway to meet the increased demand for glutathione and phytochelatins during heavy metal stress (Louie et al., 2003).

CHAPTER IV

Differential expression of *Arabidopsis* sulfurtransferases under various conditions

Abstract

Sulfurtransferases (Str) comprise a group of enzymes widely distributed in archaea, eubacteria, and eukaryota which catalyse the transfer of a sulfur atom from suitable sulfur donors to nucleophilic sulfur acceptors. Neither the *in vivo* sulfur donors nor the acceptors of Str could be clearly identified in any of the organisms investigated so far. In *Arabidopsis thaliana* 20 Str proteins have been identified and grouped according to sequence homology. To investigate their respective *in vivo* function, *Arabidopsis* plants were grown in sterile hydroponic cultures at different sulfate (0.05, 0.5 and 1.5 mM) and phosphate concentrations (0.1 and 1 mM), and in medium supplemented with 1 mM thiosulfate (TS). Northern blot analysis revealed the differential expression of the Str investigated. TS Str activity was significantly increased at low sulfate concentrations in the medium. The *Str* mRNA levels were highly dependent on the developmental stage of the *Arabidopsis* plants. The expression of most Str analysed increased with progressing plant age in parallel with increasing 3-mercaptopyruvate and TS Str activities. The Str investigated were differentially expressed in a light/dark cycle whereas Str enzyme activities were not affected by the light conditions. The results indicate that each Str is regulated in a different way and plays an individual specific role in the plant metabolism.

Introduction

Sulfurtransferase/rhodanese (Str) enzymes catalyse the transfer of a sulfur atom from suitable sulfur donors to nucleophilic sulfur acceptors. The most studied and best characterised Str is bovine rhodanese (EC 2.8.1.1) which catalyses, *in vitro*, the transfer of a sulfane sulfur atom from thiosulfate (TS) to cyanide, leading to the formation of sulfite and thiocyanate (Westley, 1973). In most organisms analysed so far Str are encoded by small gene families. All gene products are characterised by a short rhodanese (Rhd) pattern and by a well-defined Rhd domain. Rhd domains are ubiquitous structural modules that can be found as tandem repeats, with the C-terminal domain hosting the active-site cysteine (Cys) residue, as single domain proteins or as members of multi domain proteins

(Bordo & Bork, 2002). Recent results show that Rhd domains are structurally related to the Cdc25 phosphatase catalytic domain and may share a common evolutionary origin (Hofmann et al., 1998; Bordo & Bork, 2002; Forlani et al., 2003). In the *Arabidopsis thaliana* genome 18 putative Str (AtStr) were identified and classified into six groups according to amino acid sequence homologies (Bauer & Papenbrock, 2002). Recently, two additional Str could be found in the databases with similarity to group II (AtStr4a, At3g25480) and group VI proteins (AtStr17a, At2g21045) (Bartels & Papenbrock, unpublished results). Group I proteins belong to the two-domain Str, whereas all other Str contain only one Rhd domain, some in combination with another distinct protein domain. Single-domain Str in group VI show the highest similarity in the catalytic site in comparison to the active site of single-domain Str in other organisms such as *Escherichia coli* (Ray et al., 2000). All other AtStr proteins containing a Rhd domain show a high divergence with respect to the catalytic site, length and additional sequence patterns among each other. In some sequences even the catalytically active Cys residue is replaced by another amino acid. Thus the persulfide formation between the thiol group and the sulfur donor observed in bovine Rhd (Westley, 1973) does not occur in all putative Str and alternative substrates have to be postulated.

It was shown previously that two-domain AtStr proteins possess *in vitro* 3-mercaptopyruvate (3-MP) and TS Str activity, preferring 3-MP over TS, whereas the single-domain Str investigated show a high substrate specificity only for TS (Bauer & Papenbrock, 2002). Further experiments need to be done to verify the differences of *in vitro* substrate specificities of the proteins and to obtain evidence for their activities *in vivo*. The results will help to classify the proteins' role in the total (sulfur) metabolism in plants. Apart from the analysis of *Str* mutants planned for the near future, wild-type plants need to be analysed for *Str* expression and activity, and metabolite contents under various conditions.

So far, only the expression of *AtStr1* and *AtStr2* (Papenbrock & Schmidt, 2000a, 2000b; Meyer et al., 2003) and *AtStr15*/*SEN1* (Chung et al., 1997; Oh et al., 1996; Schenk et al., 2005; Weaver et al., 1998; Yu et al., 2005) was analysed under various conditions. By expression and promoter analysis *AtStr15* was shown to be regulated by many different factors. The observed dark-induced activation is due to the release of the suppression of the *AtStr15* promoter activity by sugar (Chung et al., 1997; Fujiki et al., 2001). Phosphate deficiency strongly induced the *AtStr15* promoter in roots whereas potassium and nitrogen deficiency did not influence promoter activity. Abiotic stresses, especially drought,

induced the *AtStr15* promoter. Different hormones enhanced promoter activity in various organs (Oh et al., 1996; Chung et al., 1997; Yu et al., 2005). It was further concluded from expression studies that the *AtStr15* promoter is regulated by signals that link plant defence responses and senescence (Schenk et al., 2005). Although a lot of interesting data has been collected the biological function of AtStr15 is still unknown; therefore these experiments need to be extended by functional approaches.

To investigate whether sequence homology of AtStr in one group also implicates similar function, comparative expression analysis paralleled by the determination of enzyme activities and metabolite contents in plant extracts need to be determined.

Many enzymes involved in the sulfur metabolism are regulated by the availability of sulfate and Cys, the first organic compound containing highly reduced sulfur. Therefore the sulfur nutritional status was investigated in controlled feeding experiments. The sequence similarity of Str to phosphatases raises the question of a possible impact of different phosphate concentrations on activity and expression of Str. Substrate induction is a common phenomenon and the analysis of the induction of expression and enzyme activity of Str by TS might help to differentiate the function of two-domain 3-MP and TS Str from that of single-domain TS Str. The analysis of influences of plant development and light conditions on Str expression and activity complete this experimental study.

Material & Methods

Growth, treatment and harvest of plants

Seeds of *Arabidopsis thaliana*, ecotype Col-0 or C24, were originally obtained from the Arabidopsis Biological Resource Center (ABRC). Seeds were germinated on substrate TKS1 (Floragard, Oldenburg, Germany). Seedlings 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 µmol x m^{-2} x s^{-1} (sodium vapour lamps, SON-T Agro 400, Philips). After 3 weeks the plants were transplanted in pots (diameter 7 cm) with TKS2 (Floragard) and transferred to climatic chambers or remained in the greenhouse.

To investigate the influence of various nutrient concentrations, *Arabidopsis* seeds were germinated and grown for 20 days in a hydroponic culture system using different media under sterile conditions (Schlesinger et al., 2003). 30 mg of surface-sterilised seeds were dissolved in 100 µl sterile agarose sugar solution (2% low melt agarose, 30% sugar in H_2O) to fix the seeds on the mesh. In one experiment the sulfate concentrations were

varied: the medium contained 650 µM $Ca(NO_3)_2$, 200 µM NH_4NO_3, 300 µM KNO_3, 50 (low) or 500 µM (medium) or 1500 µM (high) $MgSO_4$, 450 µM $MgCl_2$ (only low sulfate medium), 100 µM KH_2PO_4, 8 µM H_3BO_3, 0,2 µM $ZnCl_2$, 0,1 µM $CuCl_2$, 2 µM $MnCl_2$, 0,1 µM $MoNaO_4$, and 40 µM FeEDTA. For the other experiments medium was prepared according to Murashige & Skoog (1962) and either supplemented with 1 mM thiosulfate or with phosphate concentrations of 100 µM (low) and 1 mM (high).

To investigate natural senescence, *Arabidopsis* plants were grown in the greenhouse for up to six weeks, counted from the transfer into the pots, and the parts above ground were harvested every week. The oldest leaves were comparable to the S3 stage as defined by Lohmann et al. (1994).

The influence of light and darkness on expression and activity were investigated in four-week-old plants grown in a 12 h light/12 h dark cycle in a climatic chamber at a quantum fluence rate of 50 µmol x m^{-2} x s^{-1} (TLD 58W/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 nearly one and a half day starting 1 h after the onset of light.

RNA extraction and Northern blot analysis

Total RNA was extracted essentially as described (Chomczynski & Sacchi, 1987). RNA samples (15 µg) were separated in 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 probes obtained by PCR. 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. The probes were prepared as described in Papenbrock & Schmidt (2000b) (*AtStr1* and *AtStr2*) and in Riemenschneider et al. (2005) (*AtP1*). For probes against *AtStr9* (At2g42220), *AtStr10* (At3g08920), *AtStr11* (At4g24750), *AtStr14* (At4g27700) and *AtStr15* (At4g35770), a cDNA library was used as template and primers 124 (5'-GGA TCC GCG GGG ATC ATA AGC CCT A-3') and 125 (5'-GTC GAC GCT TGT TGG AGG AAA GAG C-3') for *AtStr9*, primers 120 (5'-GGA TCC ACA GTT CTT CTT CCT CAG-3') and 121 (5'-CTG CAG ACG ACT CTT CTG GCC AAA A-3') for *AtStr10*, primers 126 (5'-GGA TCC GAG TCT CTA TCT CTT CCC-3') and 127 (5'-CTG CAG ATG GCC TCT TAA CTC CTG A-3') for *AtStr11*. Primers 118 (5'-CGG ATC CGC TTC ACT TAC TTC AAT-3') and 119 (5'-CGG TCG ACA GTC TTC TTC AAT TGT-3') for *AtStr14*, and

primers 110 (5'-CGG ATC CGA AAC CAC TGC TTT TAA-3') and 111 (5'-CCT GCA GCT CTT CTA CCG GCA GCT-3') for *AtStr15* were used for probe preparation. Primers 108 (5'-GGA TCC GCG GAC GAG AGC AGA GT-3') and 109 (5'-CCT GCA GTT GAA GAA GAA GGA GAC-3') were used for *AtStr16* (At5g66040), and primers 104 (5'-CGG ATC CTC TCA ATC AAT CTC CTC C-3') and 105 (5'-CAA GCT TAT TAG CAG ATG GCT CCT C-3') for *AtStr18* (At5g66170) with EST clones 211O17T7 and 149L5T7 as templates, respectively.

Enzyme activity measurements

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 1:5 (100 mg plant material plus 400 µl buffer). After centrifugation the protein content of the supernatant was adjusted to 1 µg x μl^{-1} to obtain equal amounts of protein in each assay sample (50 µg). The determination of Str enzyme activity using 3-mercaptopyruvate and thiosulfate, respectively, as sulfur donor was performed as described (Meyer et al., 2003).

Analysis of ions and metabolites

Sulfate and phosphate were determined by capillary electrophoresis (CE) in the following way: 30 mg of deep frozen, fine-ground plant material was solved in 700 µl HPLC grade H_2O, mixed for 1 min, incubated at room temperature for 10 min, mixed again for 1 min and centrifuged for 10 min at 13.200*g* and 4°C. The supernatant was transferred to a new reaction tube and centrifuged another 10 min. The supernatant was transferred to a 500 µl reaction tube and used for CE analysis. For analysis of the culture medium 1 ml aliquots were centrifuged for 10 min at 13.200*g* and 4°C. 500 µl of the supernatant were transferred to a 500 µl reaction tube and directly used for CE analysis.

A Beckman Coulter P/ACE^{TM} MDQ Capillary Electrophoresis System with MDQ-PDA detector (Krefeld, Germany) was used for CE analyses. Separations were performed in a Beckman Coulter eCAP^{TM} CE-MS capillary (fused silica, 50 µm i.d., 84 cm total length, 74 cm effective length). Before starting the analyses the capillary was rinsed with 0.1 M NaOH (20 psi, 10 min) and equilibrated with the background electrolyte Basic Anion Buffer for HPCE (Agilent Technologies, Waldbronn, Germany) at 14.5 psi, 10 min. Injection was done by applying 0.7 psi for 6 s. Separation of the samples was performed by applying 22 kV, reverse polarity for 15 min. After each run, the capillary was washed with the background electrolyte for 3 min. Buffer was changed after 8 to 10 runs. Samples were

detected at 350 or 217 nm with a bandwidth of 20 nm. Calibration graphs for sulfate and phosphate were generated with 78 to 10,000 µM $(NH_4)_2SO_4$ and 78 to 5,000 µM KH_2PO_4. Elaboration of the electropherograms was done using Karat 32 7.0 software. Cysteine, glutathione and thiosulfate were determined by HPLC analysis as described (Riemenschneider et al., 2005).

Miscellaneous and statistical evaluation

Protein estimation was performed according to Bradford (1976) using bovine serum albumin as a standard. Each experiment was repeated three times. Each type of analysis was done in duplicate with the same frozen plant material. Representative Northern blots are shown. Statistical analysis of enzyme activity and metabolite contents was performed using the Student t-test (SigmaPlot for Windows version 8.0).

Results

Nutritional influence on sulfurtransferase expression and activity

The influence of different concentrations of sulfate, phosphate, TS and D- and L-Cys in the medium on the expression and activity of Str was investigated. A sterile hydroponic system (Fig. 1) was used to be able to strictly control the environmental conditions. The *Arabidopsis* ecotype Col-0 was selected after pre-tests including half a dozen ecotypes on account of even growing. On average the plants possessed six primary leaves at harvesting. The different conditions chosen did not cause significant differences in growth, also the root to shoot ratios were not impaired (data not shown). Originally it was planned to separate roots and shoots for further analysis, however, even after selecting the most suitable *Arabidopsis* ecotype with a relatively large root mass the biomass produced during the experiments was not sufficient to conduct all experiments planned. In comparison to the medium prepared according to Murashige & Skoog (MS) (1962) used in variation of the phosphate concentration and the TS treatment, the medium used in the sulfate experiment contained much higher salt concentrations, especially of nitrogen salts. Therefore, the different treatments should be compared only to their respective control.

Fig. 1 Hydroponic culture of *Arabidopsis* Col-0 in liquid medium in a glass jar. Seeds were sown sterile on a metal mesh just reached by the medium. Plants were grown sterile for 20 days in a climatic chamber at 22°C in a 12h/12h light/dark rhythm.

The effects of different sulfate concentrations in the medium were investigated by germinating seeds in medium with 50 (low), 500 (medium) or 1500 µM (high) sulfate concentrations and growing them for 20 days. The *Arabidopsis* plants grown at different sulfate concentrations were phenotypically identical. The lowest sulfate concentration was chosen because it represents the threshold for normal growth rates and should reflect the conditions on the field of sulfur-fertilised and non-fertilised *Brassica napus* plants (pers. communication, Prof. Dr. Dr. E. Schnug, Braunschweig, Germany). In pre-tests suitable TS concentrations were tested (up to 5 mM). In the higher concentration range the plants grew much slower and showed small necrotic spots on their leaves. Finally, a concentration of 1 mM was chosen because it was the highest concentration with no symptoms visible. The different phosphate concentrations had no influence on the phenotype. In pre-tests also the highest concentration of D- and L-Cys (200 µM) not causing visible symptoms was determined.

Northern blot analysis was conducted using probes directed against Str from group I (*AtStr1* and *AtStr2*), IV (*AtStr9, AtStr10* and *AtStr11*), and VI (*AtStr14* , *AtStr15*, *AtStr16* and *AtStr18, AtStr17* seems to be a pseudogene) (Fig. 2A). *AtStr1* was induced at the low sulfate concentration whereas *AtStr2* levels were not affected. The expression of all three members of group IV was not altered by the different sulfate concentrations used. *AtStr14* showed decreased mRNA levels at 50 µM sulfate while *AtStr15* expression was clearly induced by low sulfate in the medium. The expression of the *AtP1* gene can be used as a marker for oxidative stress. At all three concentrations chosen, 50, 500, 1500 µM, it was expressed, however, at 50 µM there was a significant increase in the expression level.

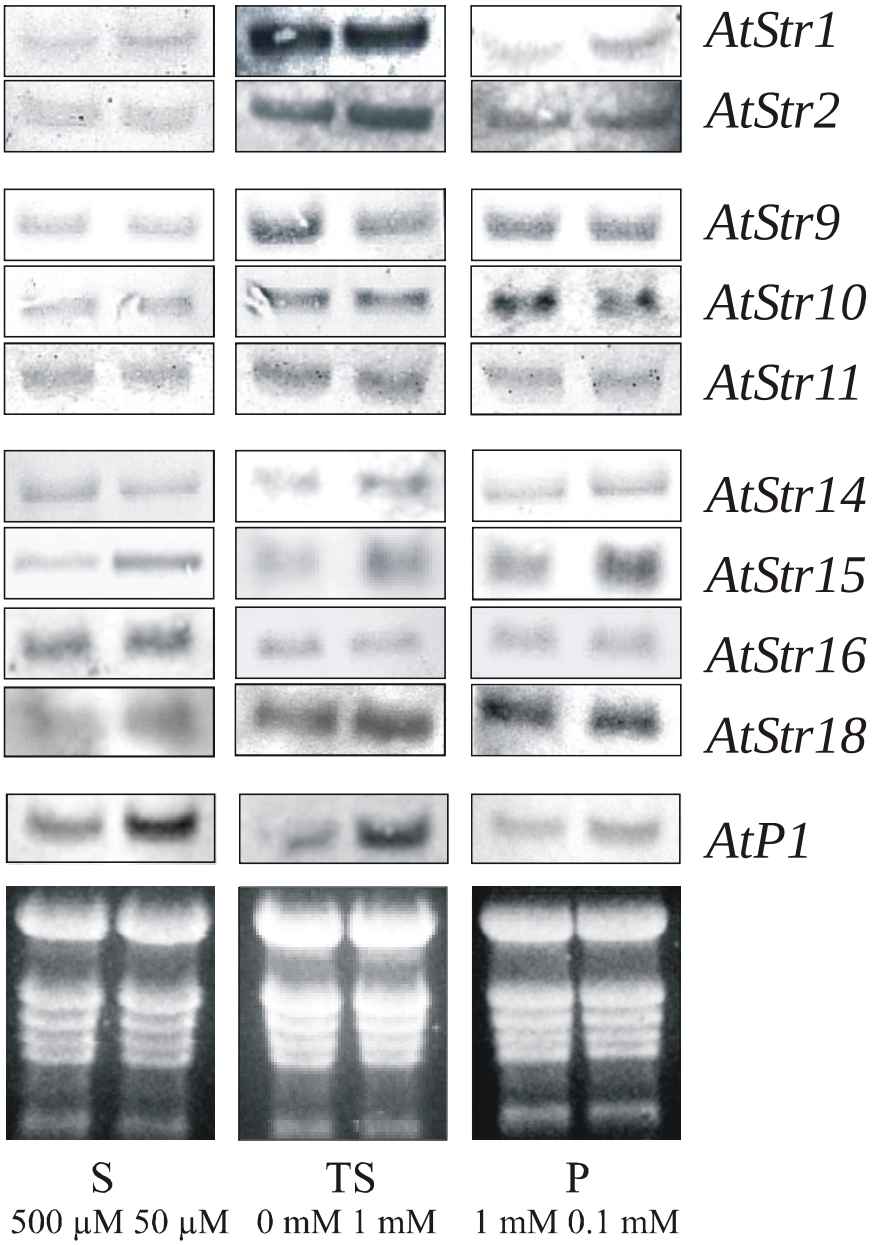

Fig. 2 (A) Northern blot analysis of *AtStr* and *AtP1* transcripts in *Arabidopsis* Col-0 plant material grown in liquid medium depleted of or supplemented with sulfate (S) (500 µM/50 µM), thiosulfate (TS) (0 mM/1 mM) and phosphate (P) (1 mM/0.1 mM) for 20 days. All 'above ground' parts of the plants were harvested, total RNA was extracted and 15 to 30 µg RNA were loaded in each lane and blotted. To ensure equal loading of the extracted RNA representative ethidium bromide-stained gels are shown at the bottom. Blots were probed with digoxigenin-labelled cDNAs. *AtP1* was used as a marker for oxidative stress.

Further, the influence of TS as a putative substrate in the growth medium was investigated. *AtStr2* expression was induced by TS whereas that of *AtStr1* remained unchanged. In group IV only the expression of *AtStr9* was influenced by TS; its expression was reduced. In group VI *AtStr14*, *AtStr15*, and *AtStr18* mRNA levels were slightly increased by

addition of 1 mM TS. Also the *AtP1* levels were elevated at 1 mM TS indicating slight oxidative stress.

In addition, the influence of high (1 mM) and low (100 µM) concentrations of phosphate in the medium was surveyed. *AtStr1* mRNA levels were slightly increased at low phosphate. Expression of group IV members was almost unchanged. *AtStr14* and also *AtStr15* levels were slightly increased at low phosphate conditions. The marker gene for oxidative stress showed a very small increase at 0.1 mM phosphate in comparison to 1 mM.

In summary, it can be concluded that the expression of most of the Str investigated are neither significantly influenced by different sulfate and phosphate concentrations in the medium nor induced by the addition of TS. The *AtStr15* gene plays an exceptional role since its expression is increased at the respective stress condition, i.e. low sulfate, low phosphate and additional TS.

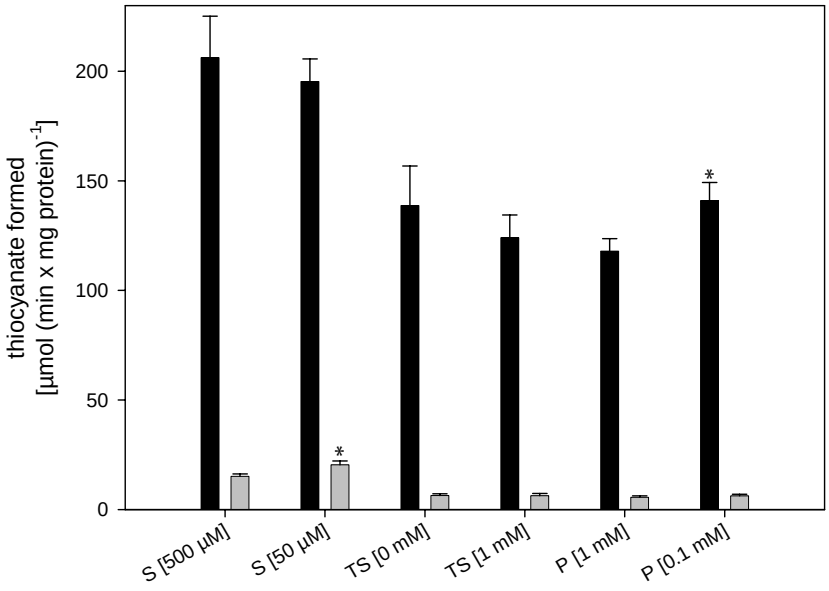

Fig. 2 (B) Sulfurtransferase enzyme activity measurements in *Arabidopsis* Col-0 plant material grown in liquid medium depleted of or supplemented with sulfate (S) (500 µM/50 µM), thiosulfate (TS) (0 mM/1 mM) and phosphate (P) (1 mM/0.1 mM) for 20 days using 3-mercaptopyruvate (black bars) and thiosulfate (grey bars) as substrates were done. All 'above ground' parts of the plants were harvested. Specific activities significantly different to the respective control at $p < 0{,}001$ are marked with an asterisk.

The 3-MP Str activity was significantly ($p < 0{,}01$) changed only by the low phosphate concentration (Fig. 2B); the TS Str activity was significantly increased at low sulfate ($p < 0{,}001$). Addition of TS did neither influence the 3-MP nor the TS enzyme activity.

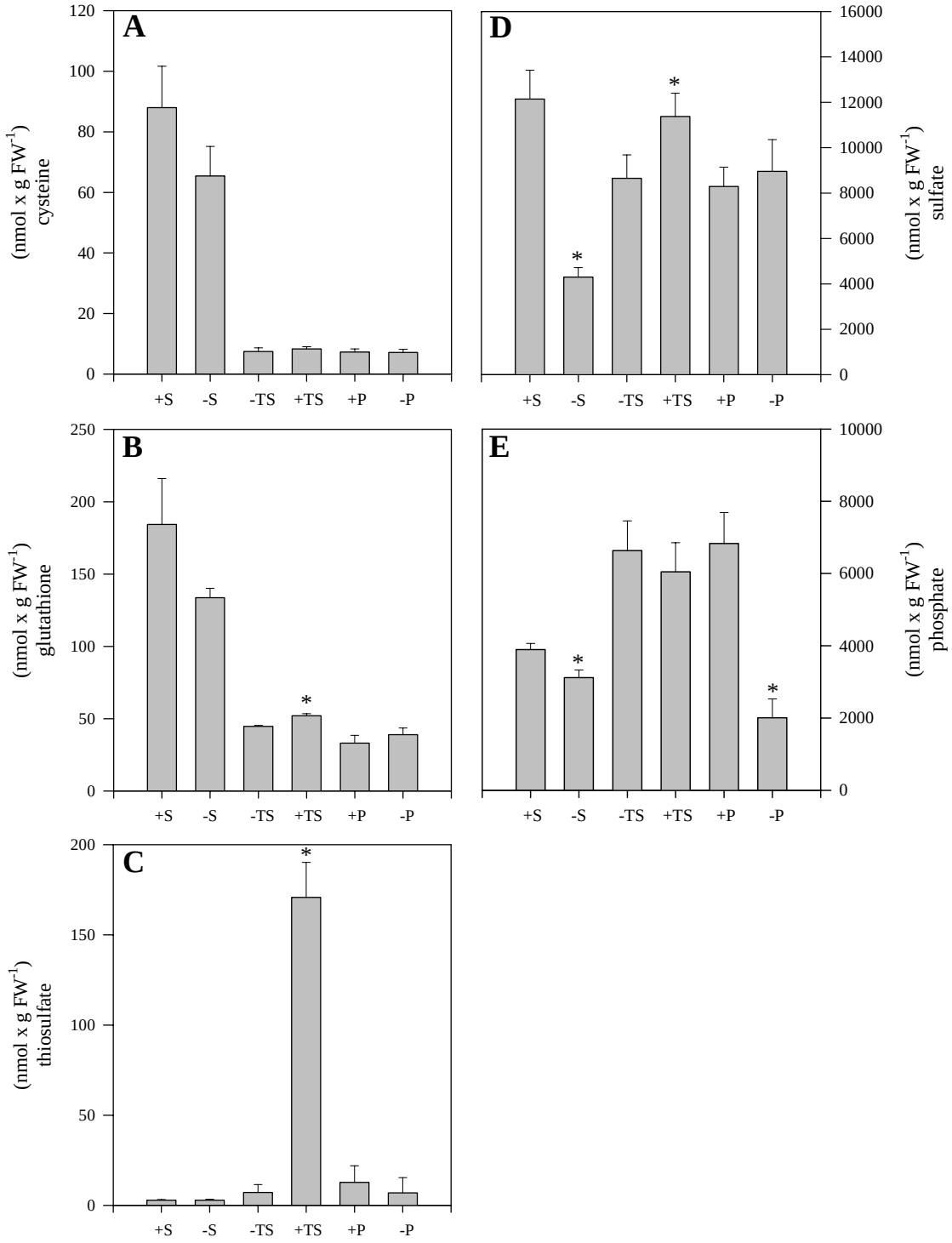

Fig. 3 Metabolite and ion contents of plants grown under different nutritional conditions. The same plant material as described in Fig. 2 was investigated. Cysteine, glutathione and thiosulfate were determined by HPLC, sulfate and phosphate by capillary electrophoresis. The metabolite and ion contents significantly different to the respective control at $p < 0{,}001$ or $p < 0{,}010$ are marked with an asterisk. P, phosphate; S, sulfate; TS, thiosulfate.

In the same plant material Cys, glutathione and TS contents were determined by HPLC, and sulfate and phosphate contents by capillary electrophoresis (Fig. 3A-E). To avoid perturbation of the measurements by metabolites from the medium the plant parts above the metal net (Fig. 1) did not have any contact with the liquid medium. Also during harvesting contact between medium and plant material was avoided. The Cys and glutathione contents were reduced by shortened sulfate supply in comparison to the controls. Addition of TS significantly enhanced glutathione content.

The TS content was significantly increased in the plants grown on TS-rich medium. The sulfate content in plants grown at high or low sulfate varied significantly. Also the addition of TS to the medium led to a significant increase of sulfate. Plants grown at the 10 times lower phosphate concentration contained about four times less phosphate in comparison to the plants grown at high phosphate.

Additionally, the seedlings were grown in medium supplemented with 200 µM L- and D-Cys, respectively. In comparison to the controls the plants accumulated eight times (addition of L-Cys) or 12 times (addition of D-Cys) more L-/D-Cys (data not shown). The Cys pool measured was not separated into L- and D-Cys fractions, however, the results indicate that L- as well as D-Cys was taken up by the plants. The glutatione contents were significantly raised only in the plants grown on L-Cys. The TS levels were about six times higher in L-Cys treated plants whereas D-Cys did not cause any change in TS content. The incubation with L-Cys led to increased sulfate levels in comparison to untreated plants whereas 200 µM D-Cys reduced the sulfate pool. Phosphate contents were significantly reduced in D-Cys treated plants only. Taken together the results demonstrate that the metabolite and ion levels inside the cell were influenced by the different treatments.

Expression levels and enzyme activity of sulfurtransferases during plant development

Arabidopsis plants were grown in the greenhouse for up to six weeks and all plant tissue above ground was used for the analyses. Northern blot analysis showed that almost all Str are differentially expressed (Fig. 4A). The expression of *AtStr1* and *AtStr2* increased continuously and very clearly from the youngest to the oldest plants (Fig. 4A, Papenbrock & Schmidt, 2000b). *AtStr9* and *AtStr10* mRNAs were almost constitutively abundant with a slight increase in older plants. The third gene in group IV, *AtStr11*, showed the highest expression levels in the younger plants and signals were almost undetectable in the later developmental stages. All members of group VI which are annotated as senescence-associated genes display diverse expression patterns. *AtStr14* mRNA levels had a

maximum of accumulation at week three and four. *AtStr15* showed strong mRNA accumulation from week three to week six. Expression of *AtStr16* continuously increased with age. *AtStr18* mRNA was only detectable in five- and six-week-old plants revealing the highest similarity to a senescence-associated gene.

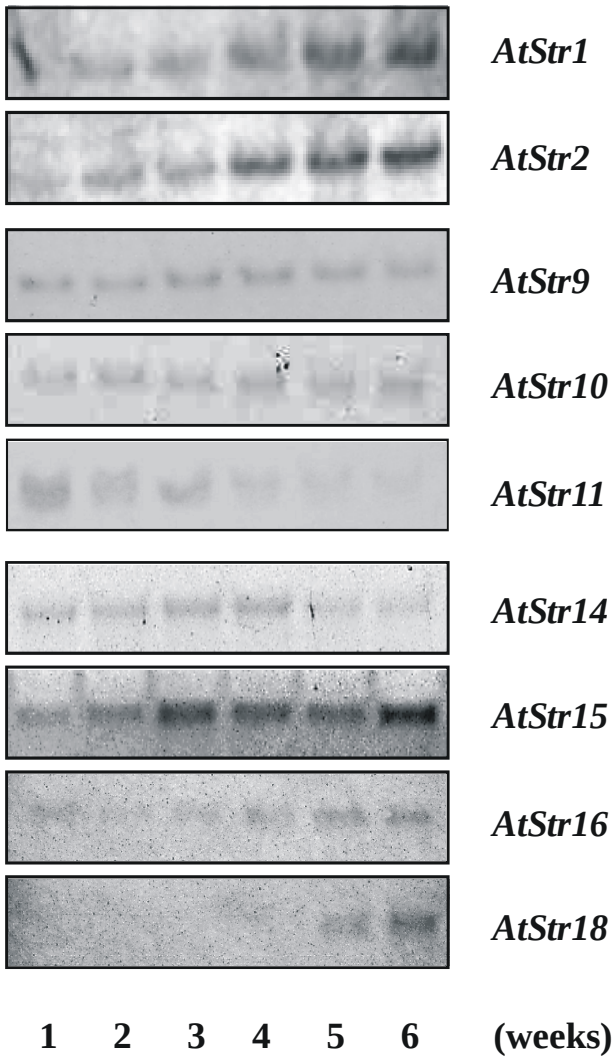

Fig. 4 (A) Northern blot analysis of *AtStr1* transcripts in *Arabidopsis* C24 during aging. Plants were grown in soil in the greenhouse. 'Above ground' parts of the plants were harvested weekly over a period of 6 weeks, total RNA was extracted and 15 µg RNA were loaded in each lane and blotted. To ensure equal loading of the extracted RNA a representative ethidium bromide-stained gel is shown at the bottom. Probes for hybridisation were labelled with digoxigenin by PCR.

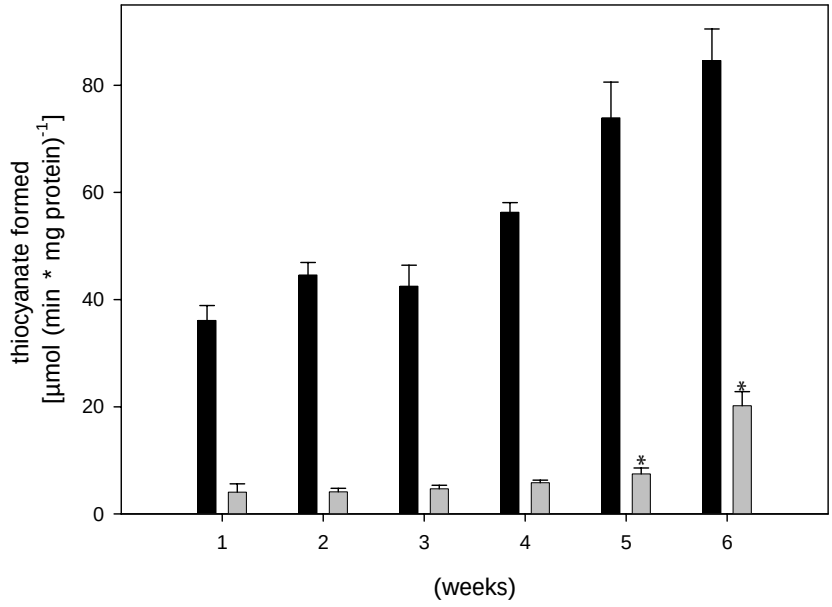

Fig. 4 (B) Sulfurtransferase enzyme activity measurements in *Arabidopsis* C24 during aging using thiosulfate (grey bars) as substrate were done. Plants were grown in soil in the greenhouse. 'Above ground' parts of the plants were harvested weekly over a period of 6 weeks. Data for enzyme activities using 3-mercaptopyruvate (black bars) were taken from Papenbrock & Schmidt (2000b). The specific activities significantly different to the respective control (one-week-old plants) at $p < 0{,}001$ are marked with an asterisk.

Both substrates, 3-MP and TS, were used in enzyme activity assays (Fig. 4B). The 3-MP Str activity increased almost continuously from younger to older plants whereas the TS Str activity was elevated in five- and six-week-old plants.

Expression levels and enzyme activity of sulfurtranferases 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. Again all Str investigated showed different expression patterns during the light/dark cycle. *AtStr1* and *AtStr2* did not show any cycling of their mRNA levels during the light/dark cycle (Fig. 5A). In group IV *AtStr9* mRNA levels decreased drastically in the dark and increased again before the light period started. Also for *AtStr10* a slight reduction in the mRNA accumulation during the dark period was observed, however, with a shift of about 4 h in comparison to *AtStr9* mRNA. None of the Northern blot results for *AtStr11* revealed a clear tendency of expression. For *AtStr14* a maximum of mRNA expression 5 h after the onset of light was observed followed by a decrease at night. *AtStr15* mRNA accumulation

decreased after the beginning of the light period and reached a maximum in the middle of the dark period. *AtStr16* mRNA levels slightly decreased in the dark and showed a small maximum 5 h after onset of the light. mRNA levels of *AtStr18* were not detectable in four-week-old plants although we tried to increase the sensitivity of our method by increasing the RNA loading amount to 25 µg per lane and by application of the more sensitive chemiluminescent detection method. The successful detection of *AtStr18* mRNA in the three-week-old plants grown in hydroponic culture indicate a higher physiological age of those plants than four-week-old greenhouse-grown plants.

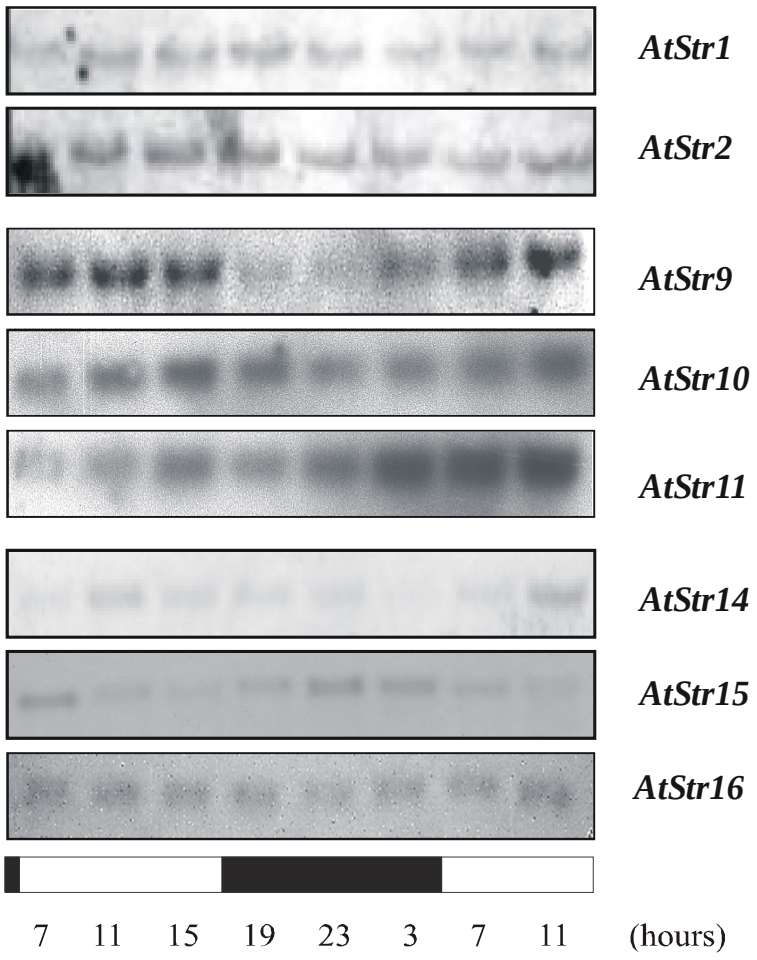

Fig. 5 (A) Northern blot analysis of *AtStr* transcripts in *Arabidopsis* C24 during diurnal light/dark cycling. Plants were grown in 12/12h light/dark rhythm for 4 weeks. 'Above ground' parts of the plants were harvested every 4 h starting 1 h after onset of light. Total RNA was extracted and 15 µg RNA were loaded in each lane and blotted. To ensure equal loading of the extracted RNA a representative ethidium bromide-stained gel is shown at the bottom. Probes for hybridisation were labelled with digoxigenin by PCR.

Neither 3-MP nor TS Str activities were changed during the light/dark cycle (Fig. 5B).

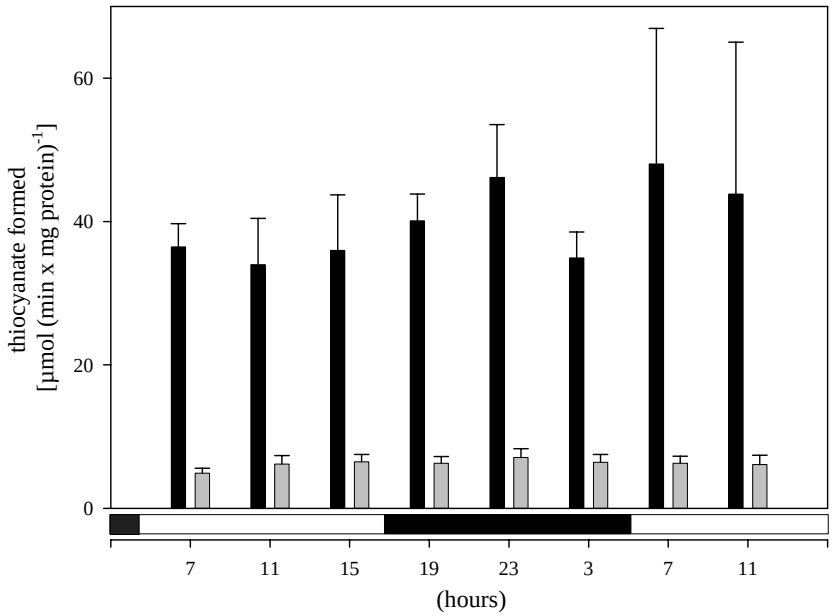

Fig. 5 (B) Sulfurtransferase enzyme activity measurements in *Arabidopsis* C24 during diurnal light/dark cycling using 3-mercaptopyruvate (black bars) and thiosulfate (grey bars) as substrates were done. Plants were grown in 12/12h light/dark rhythm for 4 weeks. 'Above ground' parts of the plants were harvested every 4 h starting 1 h after onset of light.

Discussion

Nutrition influences sulfur metabolite levels, but not AtStr expression

The hydroponical system used is particularly suitable for analysing the effects of different media compositions under highly controlled conditions. There are a number of short-term experiments published in which the concentrations of nutrients were changed lasting from hours to several days. Usually the plants were grown in complete medium to a certain age and then transferred to the respective depleted medium (Yu et al., 2005; Misson et al., 2005). In this study long-term experiments were conducted to obtain information about the role of Str in adaptation processes to altered nutritional supply from the seedling stage to the almost fully developed plants.

It is generally assumed that a sulfate concentration of 50 µM in the medium is sufficient for normal growth. Only when biotic stress conditions are applied this sulfate concentration is assumed to be insufficient to meet the increased demand for sulfur-containing defence compounds (Rausch & Wachter, 2005). The *Arabidopsis AtP1* gene was expressed at all three sulfate concentrations used (50, 500, and 1500 µM), however, transcript levels were higher at the lower sulfate concentrations. The transcript level of

AtP1 can serve as a marker for oxidative stress. The *AtP1* mRNAs were shown to accumulate rapidly in *Arabidopsis* plants under various oxidative stress conditions, such as treatment with paraquat, t-butylhydroperoxide, diamide, and menadione (Babiychuk et al., 1995). The *AtP1* gene product is homologous to NADPH oxidoreductase sequences and recently, it was shown to act in scavenging the highly toxic, lipid peroxide-derived α,β-unsaturated aldehydes (Mano et al., 2002). Although phenotypically the lowest sulfate concentration did not cause any effects, such as reduction in growth, or typical sulfur deficiency symptoms, such as chlorosis of intercostal areas, our results indicate that low sulfate does impair the metabolism. The increase in thiol contents (Fig. 3) in plants grown at high sulfur concentration might either be a consequence of the higher availability of sulfur or a reaction to oxidative stress. Interestingly, the TS Str activity increased significantly at low sulfur concentration. One could assume that the TS available in the medium is degraded to thiocyanate and sulfite, and sulfite is reassimilated to Cys to fill up the Cys pool for further biosynthetic pathways.

Group I Str show TS and 3-MP Str activity *in vitro* whereas neither the *in vitro* nor the *in vivo* enzyme activity of group IV proteins is known so far. Because of insolubility of the respective recombinant proteins *in vitro,* activity was not yet demonstrated for the three proteins in group IV and AtStr14 and AtStr15 in group VI. AtStr16 and AtStr18 display TS Str activity *in vitro* (Bauer & Papenbrock, 2002). For the analysis of the influence of TS on expression and activity of AtStr *in vivo,* TS was added to the growth medium. As a putative substrate of Str an induction of *AtStr* and increased enzyme activity might be expected. However, neither expression of the *Str* investigated nor Str activity was significantly affected. Probably constitutively expressed Str provide translation products sufficient for the catabolism of additional TS.

In silico studies revealed a close structural relation of the Rhd domain to the catalytic domain of Cdc25 phosphatases (Bordo & Bork, 2002). Recently, AtStr5 (At5g03455) was identified as tyrosine phosphatase using *p*-nitrophenyl phosphate as substrate (Landrieu et al., 2004). The mutagenic elongation of the catalytic loop of the *Azotobacter vinelandii* RhdA changed the selectivity from sulfur- to phosphate-containing substrates (Forlani et al., 2003), thus supporting the hypothesis that the length of the catalytic loop is crucial in substrate selectivity and Rhd domains with an active-site of seven amino acids are able to bind substrates containing phosphorus or the chemically similar arsenic, whereas Rhd-like domains with a six-amino-acid loop and Cys at the first position bind substrates containing reactive sulfur or selenium (Bordo & Bork, 2002). Therefore, experiments to investigate

the effect of different phosphate concentrations on Str activity and expression were set up. In experimental systems, especially when using solidified culture media, it is very difficult to generate phosphate limitation conditions (Karthikeyan et al., 2002). The availability of phosphate in soil often is below 10 µM. To a certain extent plants can adapt very well to the prevailing concentration. To prove our experimental system the expression of *Sqd1*, generally accepted as a marker for phosphate limitation and deficiency, was determined and shown to be clearly increased at the low phosphate concentration (data not shown). Sqd1 is involved in the biosynthesis of sulfolipids that replace phospholipids in the chloroplast membrane in phosphate limiting conditions (Essigmann et al., 1998).

Summarising our results it can be assumed that phosphate availability does not significantly influence expression whereas the overall 3-MP activity was increased by low phosphate. In previous experiments comparative microarray analysis was conducted at 500 µM for optimal and 5 µM for limiting phosphate concentrations (Misson et al., 2005). The expression of one Str gene, *AtStr15*, was slightly reduced in leaves and roots at low phosphate. Also the expression of two sulfate transporters was changed in low phosphate: One gene (At3g15990; SULTR3;4, leaf group) was induced in leaves and roots one to two days after transfer into low phosphate medium whereas the other one (At3g12520, SULTR4;2, plastid localised or tonoplast) was repressed in roots (Misson et al., 2005).

Several recombinant AtStr proteins, AtStr1, AtStr2, AtStr7, AtStr14, AtStr16, and AtStr18, were tested for phosphatase activity using 3-*o*-methylfluorescein-phosphate as substrate. None of the proteins revealed phosphatase activity under the conditions used (Hartmann & Papenbrock, unpublished results). The sequence similarities of some putative Str to Cdc25 phosphatases do not help to identify substrates of AtStr. Probably the similarity of the catalytically active sites of the proteins investigated so far is not high enough.

Group-specific expression during plant development

The first Str identified were found to be senescence-associated (Oh et al., 1996). Consequently most of the *Arabidopsis* Str in group I and group VI have been annotated as senescence-associated proteins. With the exception of AtStr14 all Str in both groups show increased expression with progressing plant age, whereas AtStr9, AtStr10 and AtStr11 of group IV show no changes or a reverse expression pattern. In parallel with age the total Str enzyme activity using 3-MP and TS increased. The expression levels of the developmentally regulated AtStr are also increased by ethylene or its precursor 1-

aminocyclopropane-1-carboxylic acid (Meyer et al., 2003; data not shown). Further, microarray analysis revealed drastically increased *AtStr15* levels in senescent cell suspension cultures of *Arabidopsis* ecotype Ler-0 (Swidzinski et al., 2002). Mobilisation of reduced sulfur from older leaves to younger leaves or seeds is therefore an experimentally well supported function of Str.

The expression of several AtStr is regulated by a diurnal light/dark cycle

Most of the *AtStr* are regulated by light either positively (e.g. *AtStr9*) or negatively (*AtStr15*). In future experiments it would be interesting to investigate whether the *AtStr* expression is not only controlled by a diurnal light/dark cycle but also by a circadian rhythm. The different and partially reverse transcript levels might explain that there are no differences in the specific enzyme activities determined in total plant extracts. Computer-based prediction methods revealed localisation in different compartments of the cell for the 20 putative AtStr. Several AtStr were experimentally shown to be localised in plastids, mitochondria and the cytoplasm, and AtStr not investigated in more detail yet maybe localised in further compartments e.g. peroxisomes (Bauer et al., 2004; Papenbrock et al., 2000a; Bartels & Papenbrock, unpublished results). The AtStr proteins are likely to be active in different compartments at different time points during the day to fulfil their specific function.

General conclusions

The *Arabidopsis* Str family is a very diverse protein family, only unified by the Rhd domain. So far the functional analysis has been very difficult because most of the *AtStr* genes are constitutively expressed and do not reveal large differences in their expression levels in response to numerous environmental factors tested. The expression of *AtStr15* is an exception because its expression is changed by diverse biotic and abiotic factors; in general it is induced at various stress conditions. However, so far no clear conclusions for its *in vivo* activity and function could be drawn.

In several high throughput approaches changes in the abundance of Str have been observed. For example, in an analysis of the proteome of the thylakoid membrane of *Arabidopsis* plants 242 proteins were identified, of which at least 40% are integral membrane proteins. The functions of 86 proteins are unknown. Four of these proteins contain a Rhd domain: AtStr4 (At4g01050) AtStr4a (At3g25480), AtStr9 (At2g42220),

and AtStr14 (At4g27700) (Peltier et al., 2004). This kind of analysis accelerates the localisation studies of all AtStr and helps to identify putative functions.

Recently, a Str protein with similarity to AtStr17 (At2g17850) has been found in the phloem of cucurbita plants indicating a role of Str in the transfer of reduced sulfur to compounds transported in the phloem (Walz et al., 2004).

In a suppressive subtractive hybridisation approach several cadmium-induced cDNAs from the cadmium-tolerant *Datura innoxia* were enriched. One of four cadmium-specific cDNAs had homology to the AtStr9 protein (At2g42220) (Louie et al., 2003). Further functional analysis of the proteins identified has not been done so far.

A chemical genetics approach was used to identify SIR1, a regulator of many auxin-inducible genes, in *Arabidopsis*. The *sir1* mutant was resistant to sirtinol, a small molecule that activates many auxin-inducible genes, and promotes auxin-related developmental phenotypes. SIR1 is predicted to encode a protein composed of a ubiquitin-activating enzyme E1-like domain and a Rhd domain originally annotated as molybdopterin synthase sulphurylase (AtStr13, At5g55130) (Zhao et al., 2003). However, the *in vivo* activity and function of the protein are not known.

These experimental approaches indicate that the more experimental data about AtStr is collected the more diverse possible roles in the organism become obvious. The results reveal that each single protein needs to be individually analysed in detail. Some but not all AtStr mutants analysed so far show a phenotype distinct from wild-type under normal growth conditions (Bartels & Papenbrock, unpublished data). Probably there is some redundancy in function, and double or triple knock-out or RNAi mutants have to be generated to identify the *in vivo* functions.

CHAPTER V

Characterisation of *Arabidopsis* sulfurtransferase T-DNA knock-out mutants and transgenic *Nicotiana* plants overexpressing *AtStr1*

Abstract

Sulfurtransferases (Str) are a group of enzymes widely distributed in archaea, bacteria and eukarya, that catalyse the transfer of sulfur from suitable sulfur donors to nucleophilic sulfur acceptors. However, *in vivo* substrates could not be identified unambiguously thus far. Therefore, despite the presence of Str activities in many living organisms, specific biological roles for most members of the Str / rhodanese multi protein family have not been established yet. To investigate the *in vivo* function of four *Arabidopsis thaliana* Str (AtStr) proteins, *Arabidopsis* T-DNA insertion mutants of *AtStr1*, *AtStr2*, *AtStr11* and *AtStr15* have been characterised. Additionally transgenic *Nicotiana tabacum* lines overexpressing *AtStr1* have been generated and characterised. Of the T-DNA insertion mutants *atstr2* and *atstr11* expressed a phenotype distinct from the wild-type displaying misshaped first leaves and stunted growth, respectively, even under standard growth conditions. A reduction in 3-mercaptopyruvate (3-MP) Str activity and in sulfur metabolite content could be observed in both mutants. This was surprising since AtStr11 lacks the catalytically important cysteine (Cys) residue in the active site loop and the protein thus is considered inactive concerning Str activity. AtStr2 might act in Cys catabolism, while AtStr11 is suggested to have a function in supporting the activity of other Str proteins since it is not active itself but total 3-MP Str activity is reduced in *atstr11* mutants. However, the *Arabidopsis* T-DNA insertion mutants have not been finally verified yet. A biological role of AtStr15 in molybdenum cofactor biosynthesis can be excluded while first results indicate a role of AtStr1 in plant defence reactions: tobacco plants overexpressing *AtStr1* inoculated with *Pseudomonas syringae* pv *tabaci* showed less severe chlorosis and necrosis symptoms than wild-type plants. The aim of research is the functional characterisation of all members of the Str multi protein family in *Arabidopsis*. The generation and characterisation of transgenic plants is a very useful tool for the functional characterisation of the proteins.

Introduction

All members belonging to the large and heterogeneous sulfurtransferase (Str) / rhodanese (Rhd) multi protein family contain one or more Rhd domains or signatures in their amino acid sequence. Str proteins catalyse the transfer of a sulfur atom from a suitable sulfur donor to a nucleophilic sulfur acceptor. In spite of their ubiquity, the determination of the role(s) of Str in biological processes is still largely debated since the identification of the *in vivo* sulfur donor(s) and acceptor(s) has proven elusive thus far for all organisms investigated.

In *Arabidopsis* 20 gene sequences encoding putative Str each containing at least one Rhd domain or signature have been identified by database searches. The corresponding proteins have been classified in six groups according to amino acid sequence homologies (see Table 1 Chapter I). In this part of the work, AtStr1 and AtStr2 of group I, AtStr11 of group IV and AtStr15 of group VI have been analysed regarding possible biological *in vivo* functions.

Different specific roles of Str in biological processes have been proposed: detoxification of cyanide (Nagahara et al., 1999; Vennesland et al., 1982) and of reactive oxygen species e.g. by acting as a thioredoxin oxidase in mitochondria (Nandi et al., 2000), an involvement in sulfate assimilation (Donadio et al., 1990), transport of specific sulfur compounds (Laudenbach et al., 1991) as well as biosynthesis or repair of iron-sulfur clusters (Bonomi et al., 1977). The biosynthesis of several vitamins, enzymes and cofactors includes a step of sulfur transfer and the incorporation of sulfur into the respective substrate molecule which could be carried out by Str as was shown for the biosynthesis of thiamin and thiouridine in *E. coli* (Lauhon & Kambampati, 2000; Palenchar et al., 2000) and for the synthesis of the molybdenum cofactor (MoCo) in *E. coli* and humans (Leimkühler et al., 2001; Matthies et al., 2004). Especially single Rhd domain proteins have been associated with specific stress conditions (Bordo & Bork, 2002). Str might further activate or deactivate distinct proteins by direct transfer of sulfane sulfur and thus fulfil a regulatory role in the organism (Toohey, 1989).

In plants a function of Str in the detoxification of cyanide to the less toxic thiocyanate as was shown in mammals (Nagahara et al., 1999; Vennesland et al., 1982) was proposed. Thus far, the evidence for a role of plant Str in cyanide detoxification is rather low. Str activity was detected at comparable levels in cyanogenic and non-cyanogenic plants (Chew, 1973). These results were confirmed in the way that no correlation between cyanogenesis and Rhd activity was found (Kakes & Hakvoort, 1992). An involvement of AtStr1 in cyanide detoxification as in animals appears rather unlikely since the expression level of *AtStr1* and Str activity in total protein extracts remained unchanged in cyanide treated plants (Meyer et

al., 2003). A function of Str in providing reduced sulfur for the biosynthesis or repair of iron-sulfur clusters was investigated previously with contradictory results. The Rhd-mediated transfer of reduced sulfur to the iron-sulfur cluster of ferredoxin was demonstrated in spinach (Bonomi et al., 1977). Whereas AtStr1 seems not directly involved in iron-sulfur cluster assembly (Nakamura et al., 2000). For several AtStr genes an association to senescence and stress was postulated (Meyer et al., 2003; Papenbrock & Schmidt, 2000b). In a suppressive subtractive hybridisation approach a protein from cadmium-tolerant *Datura innoxia* with homology to AtStr9 from *Arabidopsis* was shown to be cadmium-induced and was suggested to provide a source of sulfide that is independent of the normal sulfate assimilatory pathway to meet increased demands for cysteine (Cys) respectively glutathione and phytochelatins during heavy metal stress (Louie et al., 2003). *Arabidopsis SEN1* (*AtStr15*) is induced by several senescence inducing factors including age, darkness, and phytohormones (Oh et al., 1996). In radish and tobacco dark-inducible, senescence-associated homologues of AtStr15 encoded by *Rsdin1* and *Ntdin*, respectively, were identified that accumulated upon prolonged darkness, ethylene, cytokinin and heat stress treatment (Azumi & Watanabe, 1991; Shimada et al., 1998; Yang et al., 2003). The Ntdin protein was shown to be involved in molybdenum cofactor (MoCo) biosynthesis (Yang et al., 2003). Str have been suggested to play a role during senescence in the mobilisation of sulfur for transport processes from older leaves into younger leaves or fruits (Papenbrock & Schmidt, 2000b).

It is known since antiquity that sulfur has protective effects against pests and diseases. Sulfur supply thus influences plant resistance. In a recent study the increased disease susceptibility of sulfur deficient *Brassica napus* plants towards distinct fungal and bacterial pathogens was shown to be caused at least partially by a reduction of sulfur-dependent phytoanticipins (Dubuis et al., 2005). Elemental sulfur, glutathione and H_2S are products of specific Str reactions. Besides other sulfur containing compounds like Cys-rich antifungal proteins, glucosinolates and phytoalexins, these substances play important roles in plant disease resistance (Cooper et al., 1996; Cooper & Williams, 2004; Rausch & Wachter, 2005; Williams et al., 2002; Williams & Cooper, 2003, 2004). There are hints that Str proteins might also play a role in pathogen defence reactions of the plant. In a differential display analysis aiming to isolate genes related to resistance towards the powdery mildew fungus *Erysiphe graminis* in wheat (*Triticum aestivum* L.) a wheat Str gene displaying similarity to AtStr1 and *Datisca glomerata* (Presl) Baill. thiosulfate (TS) Str was identified that might be involved in pathogen resistance against *E. graminis* in wheat (Niu et al., 2002). Recently, a Rhd-like protein displaying similarity to AtStr17 was identified in phloem exudates of

Curcubita maxima Duch. by two-dimensional gel electrophoresis and subsequent mass spectrometry. The protein is suggested to be involved in stress and defence responses of the plant by acting as phytohormone and/or in signalling (Walz et al., 2004).

The high abundance of putative Str proteins in *Arabidopsis* as well as the wide variety in the amino acid composition of the active-site loop which is crucial in substrate recognition and catalytic activity (Bordo & Bork, 2002; Forlani et al., 2003), and the localisation of Str in different compartments of the cell (Bauer et al., 2004; Hatzfeld & Saito, 2000; Nakamura et al., 2000; Papenbrock & Schmidt, 2000a) suggest an involvement of the 20 AtStr proteins in distinct biological functions. The aim of the research is the functional characterisation of all members of the nuclear encoded Str multi protein family in *Arabidopsis*. The analysis of transgenic plants represents a useful tool in providing knowledge on the possible function(s) of proteins in the plant organism. This is especially true in case of the heterogeneous multi protein family of Str in *Arabidopsis*. In this work *Arabidopsis* T-DNA insertion mutants of *AtStr1*, *AtStr2*, *AtStr11* and *AtStr15* as well as transgenic *Nicotiana* plants heterologously overexpressing *AtStr1* are characterised.

Material & methods

Growth, treatment and harvest of plants

Seeds of *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) were originally obtained from the Arabidopsis Biological Resource Center (ABRC). Seeds were germinated on substrate TKS1 (Floragard, Oldenburg, Germany). Seedlings 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 µmol x m^{-2} x s^{-1} (sodium vapor lamps, SON-T Agro 400, Philips, Hamburg, Germany). Seeds of *Arabidopsis thaliana* Col-0 *atstr1* (SALK_015593), *atstr2* (SALK_010163), *atstr11* (SALK_048306) and *atstr15* (SALK_020571) T-DNA-insertion mutants were obtained from the ABRC and the Nottingham Arabidopsis Stock Centre (NASC) (Alonso et al., 2003). Mutants were selected on Murashige & Skoog (MS) medium (Murashige & Skoog, 1962) containing 86 or 8.6 mM kanamycin to obtain homozygous seeds. Seedlings were grown in the greenhouse as described above. After three weeks the plants were transplanted to pots (diameter 7 cm) or trays with TKS2 (Floragard) and transferred to climatic chambers or remained in the greenhouse. The conditions in the chambers were as follows: a 10 h light/14 h dark rhythm at a quantum fluence rate of 50 µmol x m^{-2} x s^{-1} (TLD 58 W/33; Philips) and a constant temperature of 22°C.

Nicotiana tabacum L. cv Samsun NN plants were grown on soil in the greenhouse at the conditions described above. After three weeks the plants were transplanted to pots (diameter 11 cm) with TKS2 (Floragard) and transferred to climatic chambers or remained in the greenhouse. The conditions in the chambers were as follows: a 12 h light/12 h dark rhythm at a quantum fluence rate of 50 µmol x m^{-2} x s^{-1} (TLD 58 W/33; Philips) and a constant temperature of 22°C.

Nicotiana tabacum transgenic lines were generated by leaf disk transformation of wild-type tobacco plants. An *AtStr1* construct was produced using primers 5'-GCG TCG ACT CAT GAA GAA GAT TC-3' extended by a *Kpn*I restriction site and 5'-GTG GTA CCG CCT CGA CCC TTT T-3' extended by a *Sal*I restriction site and ligated into plasmid pBinAr (Hoefgen & Willmitzer, 1992) in sense orientation. The pBinAr construct containing a marker gene conferring resistance to kanamycin was introduced into cells of *Agrobacterium tumefaciens* strain GV2260 exhibiting resistance to rifampicin. *A. tumefaciens* transmitted gene transfer was accomplished according to Horsch et al. (1985). Transgenic plants were selected on MS medium containing antibiotics and then grown as described above.

Growth and maintenance of pathogenic bacteria and inoculation

Pseudomonas syringae pv *tabaci* bacteria were maintained in glycerine culture stocks at -70°C and on Nutrient Agar (Difco/Becton Dickinson, Sparks, MD, USA) at 4°C. For preparation of inoculation suspensions bacteria were incubated overnight in 3 ml Luria Bertani (LB) medium at 28°C and 150 rpm. Overnight cultures were transferred into 50 ml LB medium and grown until OD_{600} reached 0.6. Bacteria were harvested by centrifugation for 10 min at 4,000*g*. Pellets were resolved in 10 ml $MgCl_2$ (10 mM) and the OD_{600} was adjusted to 0.2 (~10^8 bacteria x ml^{-1}). Leaves of *Nicotiana* plants were infiltrated with 1 x 10^8 cells x ml^{-1} of *Pseudomonas syringae* pv *tabaci*, virulent on *Nicotiana*, by using a syringe and infiltrating the bacterial solution by pressure from the underside of the leaf into the tissue. Four injections per leaf were carried out.

PCR analysis of mutant plants

To confirm that the *Arabidopsis* T-DNA insertion mutants selected contain the *NPTII* sequence insertion in the genome, mutant plants were screened by PCR using primers flanking the *NPTII* sequence: primer 387 (5'-ATG GCT AAA ATG AGA ATA-3') and primer 388 (5'-CTA AAA CAA TTC ATC CAG-3'). For PCR confirming *AtStr1* was

amplified in *Nicotiana AtStr1* sense plants but not in wild-type plants, primers 12 (5'-GTG GTA CCG CCT CGA CCC TTT T-3') and 9 (5'-CGC TGC AGT CAT GAA GAA GAT TCA-3') flanking *AtStr1* were used.

For crude genomic DNA extraction about 150 mg fresh or frozen plant material was mortared for 15 s with a plastic mortar for reagent tubes, 400 µl of extraction buffer A (200 mM Tris pH 7.5, 250 mM NaCl, 25 mM EDTA pH 7.5, 0.5% [w/v] SDS) was added, mixed for 5 s and centrifuged for 1 min at 13,000*g* and 4°C. 300 µl of the supernatant were transferred to a new reaction tube and 300 µl iso-propanol was added, mixed and incubated for 2 min at room temperature then centrifuged again for 5 min at 13,000*g* and 4°C. The supernatant was discarded and the pellet resolved in 300 µl 70% ethanol followed by centrifugation for 5 min at 13,000*g* and 4°C. The supernatant was discarded again and the pellet dried at 50°C in a heating block. The pellet was solved in 100 µl TE buffer pH 7.5 and subjected to PCR analysis.

The PCR 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), 5 µl of RedTaq buffer and 2.5 µl template DNA extract 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 of 60 s at 94°C, 60 s at 39°C and 60 s at 72°C. The amplified PCR fragments were subjected to agarose gel electrophoresis.

Southern blot analysis

Genomic DNA was extracted according to standard procedures (Sambrook et al., 1989) from *Arabidopsis* plant material for Southern blot analysis. DNA was digested overnight with *Xba*I and *Sal*I in case of the *atstr1* mutants, *Sal*I in case of the *atstr2* mutant and with *Pst*I and *Eco*RI in case of the *atstr11* and *atstr15* mutants, fractionated by agarose gel electrophoresis, and transferred to nylon membranes (Roth). Digoxigenin labelled probes were synthesised by PCR according to the manufacturer's protocols (Roche) using primers 387 and 388 (see PCR analysis of mutant plants). Colorimetric detection was done using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as substrates for alkaline phosphatase.

SDS-PAGE and Western blot analysis

For the determination of AtStr1 protein steady-state levels in transgenic *Nicotiana* plants, 100 mg plant material was ground with a mortar and pestle in liquid nitrogen. 500 µl sample buffer (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. 10 µg of total protein was subjected to denaturing SDS gel electrophoresis according to Laemmli (1970) and blotted onto nitrocellulose membranes (Sambrook et al., 1989). Monospecific antibodies produced against the recombinant *Arabidopsis* AtStr1 were used for immunodetection (Papenbrock & Schmidt, 2000a). Colorimetric detection was done as described for Southern blot analysis.

Enzyme activity measurements

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 1:5 (100 mg plant material plus 400 µl buffer). After centrifugation the protein content of the supernatant was adjusted to about 1 µg x μl^{-1} to obtain equal amounts of protein in each assay sample. Total protein content was estimated according to Bradford (1976) using bovine serum albumin as a standard. The Str enzyme activity using 3-mercaptopyruvate or thiosulfate as sulfur donor and potassium cyanide as sulfur acceptor was determined as described by Papenbrock & Schmidt (2000a). The incubation time was reduced to 10 min at 37°C and absorption was determined at 452 nm.

Chlorate sensitivity test

To investigate a possible role of AtStr15 in MoCo biosynthesis a chlorate sensitivity test was conducted according to Yang et al. (2003). *Arabidopsis* wild-type and *atstr15* mutant plants were germinated on 1/2 strength MS medium in a climatic chamber at 12 h light/12 h dark rhythm (see above). After 4 weeks of growth, plants were transferred to fresh 1/2 strength MS medium with or without addition of 5 mM potassium chlorate. Plant growth was visually controlled for 30 days.

Analysis of ions and metabolites

Contents of total thiosulfate, glutathione and cysteine in *Arabidopsis* wild-type and T-DNA insertion mutant plant material were estimated by HPLC analysis as described in

Riemenschneider et al. (2005) for cysteine and glutathione. Thiosulfate content was estimated in the same way using sodium-thiosulfate as standard for quantification.

Results

Confirmation of a T-DNA insertion in the genome of *Arabidopsis* mutant plants

In *Arabidopsis* T-DNA insertion lines obtained from the SALK collection the *AtStr1*, *AtStr2*, *AtStr11* and *AtStr15* genes were inactivated by interruption with the antibiotic resistance marker *NPTII*. Transgenic plants of several generations (e.g. 15.B.4 and follow up generation 15.B.4.3) were selected on medium containing kanamycin. Insertion mutants of *AtStr1* were selected on 1/10 of the standard kanamycin concentration used for selection (see Material & Methods) because all seedlings died when the standard concentration of the antibiotic was used.

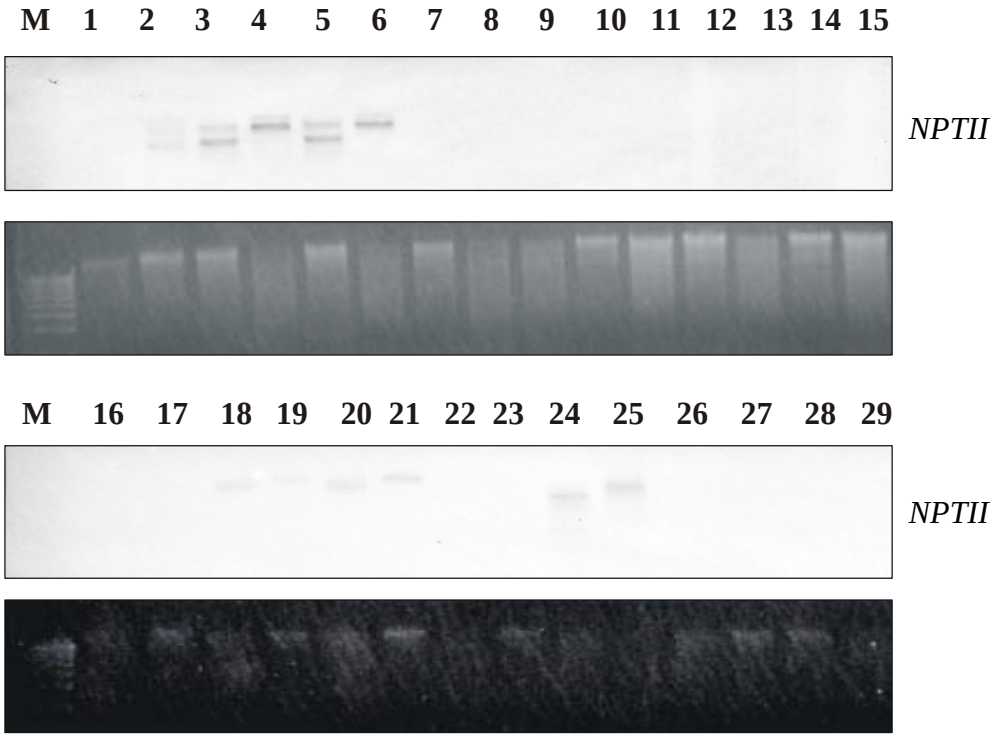

Fig. 1 Southern blot analysis of *Arabidopsis* T-DNA insertion mutants. DNA extracted from T-DNA insertion mutants and wild-type plants (wt) was digested and subjected to Southern blot analysis using a digoxigenin-labelled probe against the *NPTII* insertion sequence. **M**, DNA standard; **lane 1**, *atstr2* line 2.B.4.3.2; **2**, *atstr15* line 15.B.3.5; **3+4** *atstr15* line 15.B.4; **5+6**, *atstr15* line 15.B.4.3; **7-10**, wt; **11-14**, *atstr1* line 1.10.2; **15**, *atstr11* line 11.C.9; **16+17**, *atstr1* line 1.5.1; **18+19**, *atstr1* line 1.7.2; **20+21**, *atstr1* line 1.12.1; **22+23**, *atstr1* line 1.B.1; **24+25**, *atstr15* line 15.B.3.4; **26-29**, wt. 20 µg DNA per lane. Below the immunodetection the corresponding agarose gels are shown.

By PCR screening using primers flanking the *NPTII* insertion, three lines of *atstr1*, five lines of *atstr2*, one line of *atstr11* and five lines of *atstr15* could be identified as carrying the

NPTII T-DNA-insertion. Not in all the lines displaying a phenotype different from the wild-type under standard growth conditions the T-DNA insertion could be detected via PCR (data not shown).

In additional Southern blot analysis of digested DNA from selected mutants in two of the *atstr1* mutant lines analysed one single signal was detected (Fig. 1, lanes 18-21). No signals could be detected in *atstr2* and *atstr11* mutants while the *atstr15* mutant lines analysed were shown to carry two insertions (Fig. 1, lanes 2-6, 24+25).

Phenotypical characterisation of *Arabidopsis* T-DNA insertion mutants

When grown under standard growth conditions plants of several T-DNA insertion mutant lines of *atstr2* and of *atstr11* displayed a phenotype different from the wild-type plants (Fig. 2 and Fig.3A, B) while plants of *atstr15* insertion mutant lines did not show any phenotypical difference to wild-type plants (Fig. 3C).

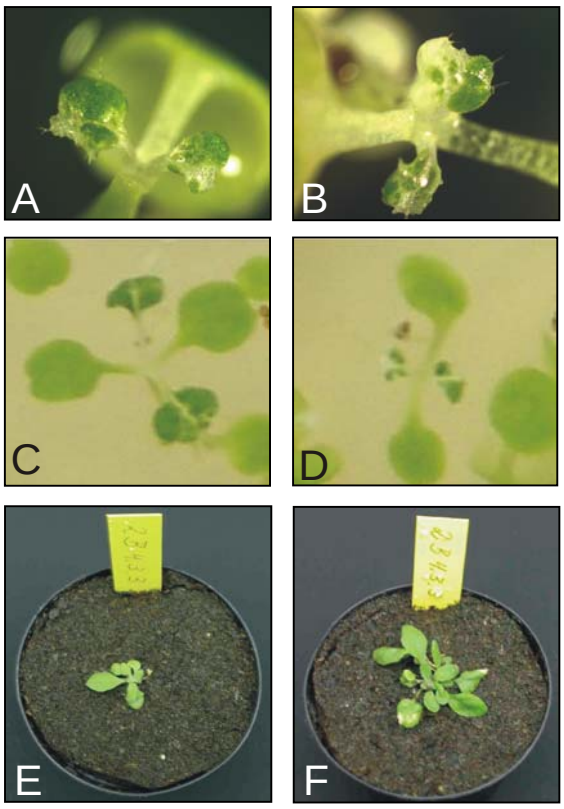

Fig. 2 *Arabidopsis* T-DNA insertion mutants *atstr2*. Deformations of first leaves in *atstr2* mutant lines: **A** and **B**, 22 dpg, 25x magnification; **C** and **D**, 37 dpg. **E** and **F**; asymmetric rosettes, 63 dpg. dpg, days past germination.

In the *atstr2* plants the first leaves showed obvious alterations in leaf formation. The laminae of the youngest leaves were misshapen. The resulting rosette of these plants was asymmetric

(Fig. 2). The alterations may not be seen anymore after some weeks of growth. The adult plants did not show phenotypical differences to the wild-type. Insertion mutants of *atstr11* displayed stunted growth (Fig. 3A, B) and sometimes only little seed production. Only small amounts of plant material could be obtained of these mutants because often fungal contamination killed the plants growing on selection medium. The *atstr11* medium plates were contaminated stronger than other plates. When grown on 1/10 of the standard antibiotic concentration the selected *atstr1* plants grew without obvious alterations compared to wild-type under standard growth conditions.

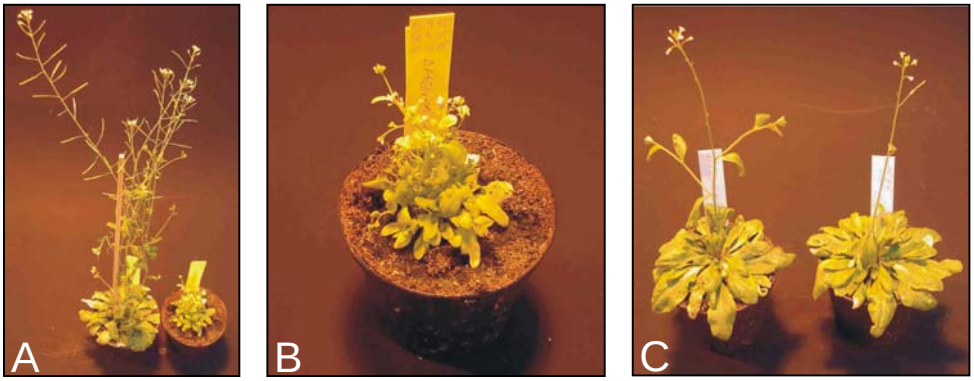

Fig. 3 *Arabidopsis* T-DNA insertion mutants *atstr11* and *atstr15*. A, wild-type (left) and *atstr11* (right) showing stunted growth, 83 dpg; **B**, *atstr11* 83 dpg; **C**, wild-type (left) and *atstr15* (right) showing no phenotypical differences under standard growth conditions, 77 dpg. dpg, days past germination.

Sulfur metabolite contents in *Arabidopsis* T-DNA insertion mutants

To check if disturbances in the sulfur metabolism can be observed in the T-DNA insertion mutants, contents of Cys, glutathione and TS in plant material of transgenic lines and wild-type plants were estimated by HPLC analysis. In Cys content no clear differences or trends were observable between *atstr2*, *atstr11*, *atstr15* and wild-type. In *atstr2* line 2.B.5 and *atstr15* line 15.B.4 the content of total Cys may be reduced compared to wild-type while in another *atstr2* and *atstr15* line as well as in *atstr11* the Cys content may be increased. The glutathione content in *atstr2* line 2.B.5 was strongly reduced compared to wild-type and the other mutants analysed. The TS content was reduced in the *atstr11* insertion mutant line and also in *atstr2* line 2.B.5 compared to wild-type content (Fig. 4).

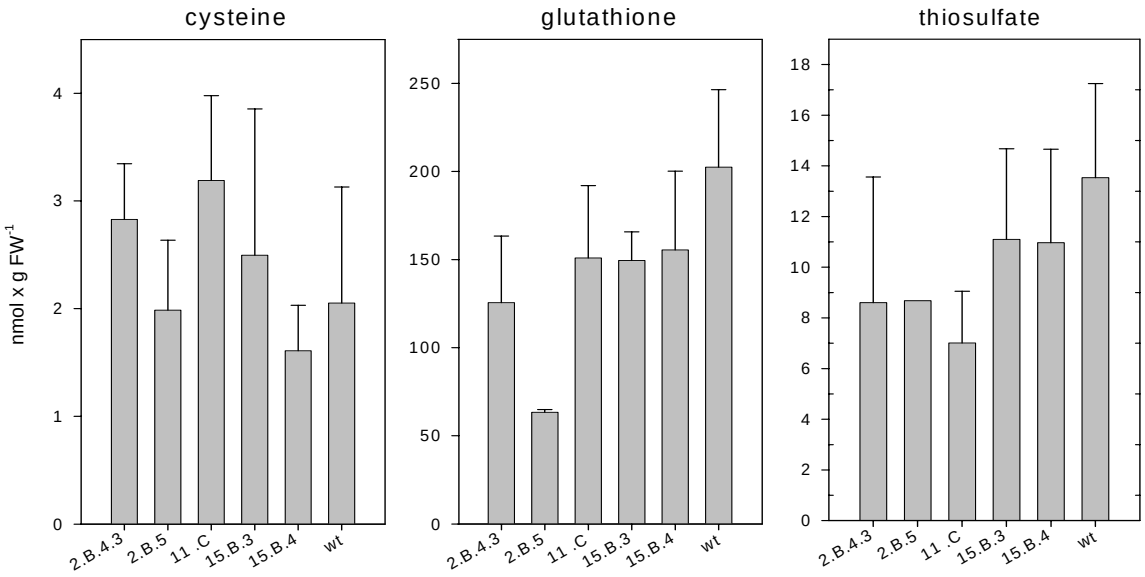

Fig. 4 Sulfur metabolite contents in *Arabidopsis* T-DNA insertion mutants. Contents of total cysteine, glutathione and thiosulfate as determined by HPLC analysis in plant material of *Arabidopsis* wild-type (wt) and T-DNA insertion mutants lines. The vertical bars denote standard deviation.

For the 2.B.5 line only one TS measurement could be done due to the little amount of plant material available. A trend of reduced contents of total glutathione and total TS is present in all the mutant lines analysed compared to the wild-type contents. The standard deviation of the three repeated measurements was quite high.

Sulfurtransferase enzyme activity in *Arabidopsis* T-DNA insertion mutants

Str enzyme activity was estimated in total protein extracts from *Arabidopsis* T-DNA insertion lines and wild-type plants using TS and 3-MP as sulfur donor substrates and potassium cyanide as sulfur acceptor substrate (Fig. 5).

When TS was used as sulfur donor, none of the mutant lines analysed showed a reduction in Str activity compared to the wild-type, while using 3-MP as sulfur donor, the *atstr2* mutant line 2.B.4.3.3 and *atstr11* line 11C*7 displayed slightly reduced Str activity compared to the wild-type. The *atstr15* insertion mutant line 15.B.3.4 displayed an increased turnover of TS by Str compared to wild-type. This is probably due to natural fluctuations normally occurring in biological material.

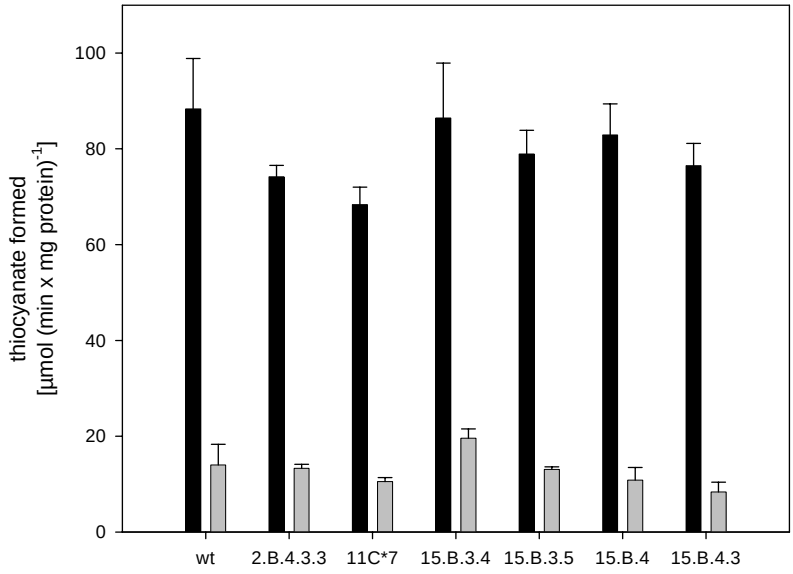

Fig. 5 Sulfurtransferase activity of *Arabidopsis* wild-type and T-DNA insertion mutant lines. Str activity was determined in total protein extracts from *Arabidopsis* wild-type (wt) and *atstr2, atstr11* and *atstr15* mutant lines using 3-mercaptopyruvate (black bars) or thiosulfate (grey bars) as sulfur donor. The vertical bars denote standard deviation.

Chlorate sensitivity of *atstr15 Arabidopsis* T-DNA insertions mutants

In a chlorate sensitivity test a possible involvement of AtStr15 in MoCo biosynthesis was investigated. Chlorate is a toxic substrate analogue of nitrate reductase. Plants deficient in MoCo biosythesis will not be able to produce functional nitrate reductase or other MoCo-proteins. Plants without functional MoCo factor and consequently without functional nitrate reductase will not be able to use chorate, while wild-type plants metabolising the toxic substrate analogue will suffer intoxication. To determine whether AtStr15 is involved in the biosynthesis of MoCo, four-week-old *atstr15* T-DNA-insertion mutants and wild-type plants were transferred to 1/2 strength MS medium containing 5 mM $KClO_3$ or fresh 1/2 strength MS medium.

After 30 days of growth on medium with or without chlorate addition no difference could be observed between wild-type and transgenic plants (Fig. 6). All of the plants, wild-type and insertion mutants, growing on medium containing chlorate showed chlorotic lesions on their laminae. These symptoms were not observed on plants growing on normal half strength MS medium without chlorate addition. There was no difference between wild-type and mutant plants concerning chlorate sensitivity. No hints for an involvement of AtStr15 in MoCo biosynthesis could be derived from the chlorate sensitivity test.

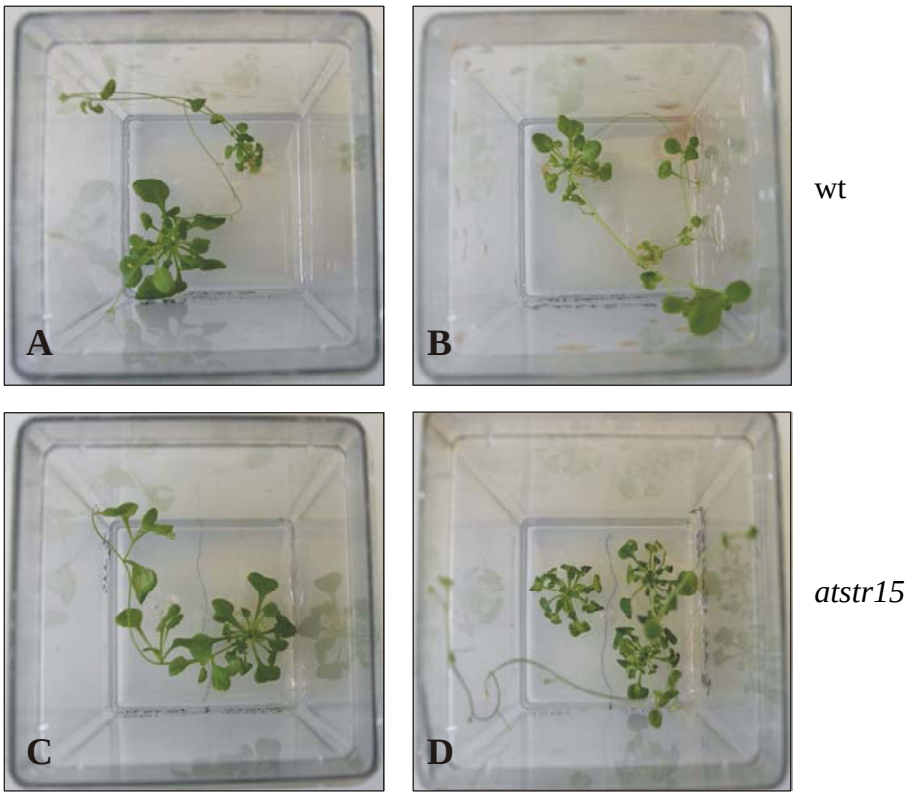

Fig. 6 Chlorate sensitivity test. *Arabidopsis* wild-type plants (wt) grown on 1/2 strength MS medium (**A**) or medium containing 5 mM $KClO_3$ (**B**) and *atstr15* T-DNA insertion mutants grown on 1/2 strength MS (**C**) and 5 mM $KClO_3$ containing medium (**D**); 30 days after transfer to $KClO_3$ containing medium.

Confirmation of *Arabidopsis AtStr1* in the genome of transgenic *Nicotiana* plants

To analyse the function of the two-domain protein AtStr1 transgenic *Nicotiana* plants containing a sense construct of the full length *AtStr1* sequence including its endogenous mitochondrial targeting peptide were generated by *Agrobacterium* transmitted transformation. PCR of *Nicotiana* wild-type and a transgenic line (103.1) expressing *AtStr1* in sense orientation confirmed *AtStr1* in transgenic but not in wild-type plants (Fig. 7).

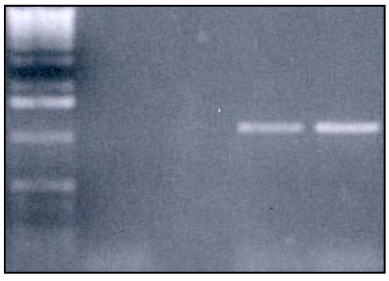

Fig. 7 PCR analysis of *Nicotiana* transgenic plants expressing *AtStr1*. PCR analysis using gene specific primers flanking the *AtStr1* sequence. DNA was extracted from wild-type (wt) and a transgenic *Nicotiana* line expressing *AtStr1* in sense orientation (103.1). M, DNA standard.

Additional Western blot analysis of *Nicotiana* wild-type and two selected transgenic lines expressing *AtStr1* in sense orientation, 103.1 and 103.9, revealed that AtStr1 from *Arabidopsis* is detectable in both transgenic lines but not in the wild-type (Fig. 8).

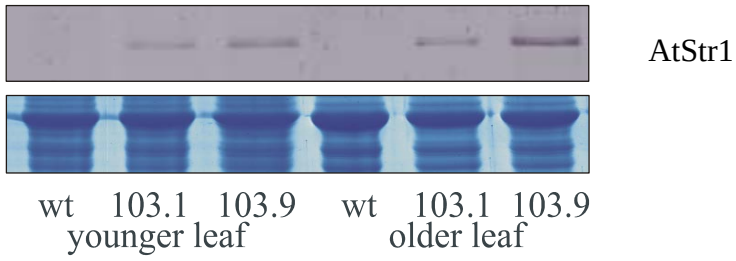

Fig. 8 Western blot analysis of transgenic and wild-type *Nicotiana*. Total protein was extracted from *Nicotiana* wild-type plants (wt) and two transgenic lines overexpressing *AtStr1* (103.1; 103.9). Plant material of two different developmental stages was harvested from four-week-old plants, separated by SDS-PAGE and blotted. For the immunodetection a monospecific antibody against AtStr1 was used. To demonstrate equal loading of the gel with protein samples, the Coomassie-stained SDS-Gel is shown below the immunoblot.

Characterisation of transgenic *Nicotiana* lines overexpressing *AtStr1*

In transgenic *Nicotiana* lines containing an *Arabidopsis* sense construct of *AtStr1* no phenotypic effect distinct from the wild-type could be observed (Fig. 9).

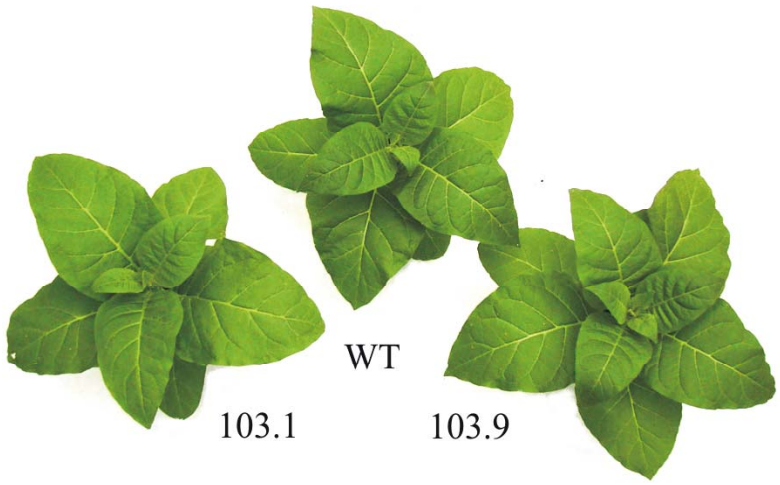

Fig. 9 Transgenic *Nicotiana* lines overexpressing *AtStr1*. *Nicotiana* wild-type (WT) and transgenic lines overexpressing *Arabidopsis AtStr1* in sense-orientation (103.1; 103.9) showing no phenotypical differences under standard growth conditions.

In Western blot analysis *Nicotiana* wild-type plants and two transgenic lines expressing *AtStr1* in sense orientation, 103.1 and 103.9, were analysed. Plant material of two different developmental stages was examined. The fifth (younger) and the ninth (older) leaf of each plant were harvested (13 leaf stage, four weeks old). AtStr1 from *Arabidopsis* was detected

in both transgenic lines but not in the wild-type with higher abundance of the AtStr1 protein in older than in younger leaves in both transgenic lines investigated (Fig. 8).

Sulfurtransferase enzyme activity in transgenic *Nicotiana* lines overexpressing *AtStr1*

The Str enzyme activity in total protein extracts from wild-type and transgenic *Nicotiana* plants heterologously expressing *Arabidopsis AtStr1* was determined using 3-MP as sulfur donor substrate and potassium cyanide as sulfur acceptor substrate. The same plant material of two different developmental stages as used in the Western blot analysis was used.

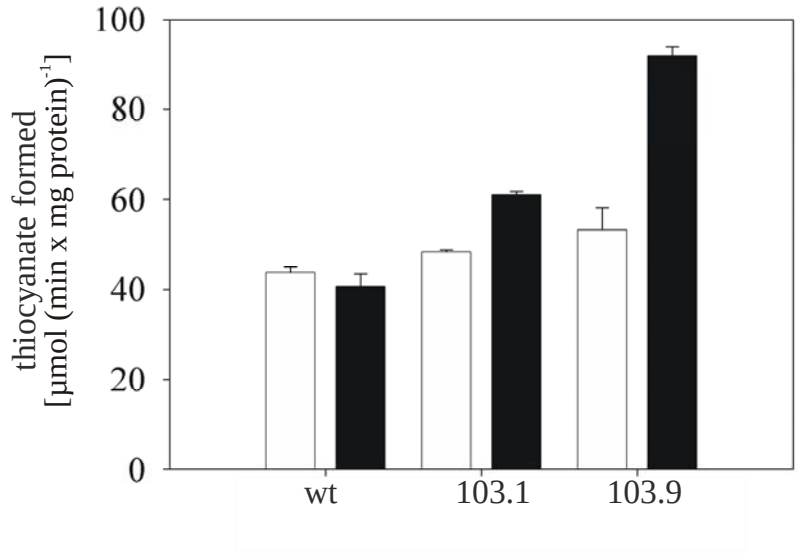

Fig. 9 Specific sulfurtransferase enzyme activities of transgenic and wild-type *Nicotiana*. Str activity was determined in total protein extracts from *Nicotiana* wild-type plants (wt) and two transgenic lines overexpressing *AtStr1* from *Arabidopsis* (103.1; 103.9) using 3-mercaptopyruvate as sulfur donor. Plant material of two different developmental stages was harvested from four-week-old plants. Open bar, younger leaf; black bar, older leaf. The vertical bars denote standard deviation.

The overexpression of *AtStr1* in *Nicotiana* resulted in increased Str enzyme activity compared to wild-type using 3-MP as sulfur donor substrate. In accordance to the Western blot analysis, in both transgenic lines the Str activity was higher in older leaves than in younger leaves, while in the wild-type plants there was no difference in Str enzyme activity between the leaves of different age (Fig. 9).

Inoculation of transgenic *Nicotiana* lines overexpressing *AtStr1* with pathogenic bacteria

To investigate a possible role of the Str proteins in the interaction of the host plant with plant pathogens, tobacco wild-type and transgenic plants overexpressing *AtStr1* from *Arabidopsis* were inoculated with *Pseudomonas syringae* pv *tabaci*, the causal agent of tobacco wildfire

(bacterial leaf blight). The bacteria enter the leaf through stomata or wounds, multiply and spread intercellularly and cause a circular yellowish-green chlorotic halo surrounding each lesion. The affected tissues in the centre of the lesion collapse and die (Agrios, 1997). The development of symptoms was monitored visually for a period of 3 weeks and Str enzyme activity was estimated in plant material from inoculated wild-type and transgenic *Nicotiana* plants using 3-MP as sulfur donor substrate. The visual bonitur of the inoculated plants clearly showed less severe chlorosis and necrosis symptoms in the transgenic lines overexpressing *AtStr1* from *Arabidopsis* compared to the wild-type (Fig. 10). At both time points analysed, 7 and 20 days past inoculation (dpi), a clear difference in the strength of chlorosis and necrosis could be observed. The symptoms developed starting from the four inoculation points on the lamina. While in the transgenic lines the necrotic lesions were restricted to the area around the inoculation points surrounded by a halo of chlorotic tissue at 20 dpi, the wild-type leaf already was almost completely necrotic. The enzyme activity results were ambiguous. No obvious trend could be observed (data not shown). The data were derived from only one experiment and are therefore only preliminary.

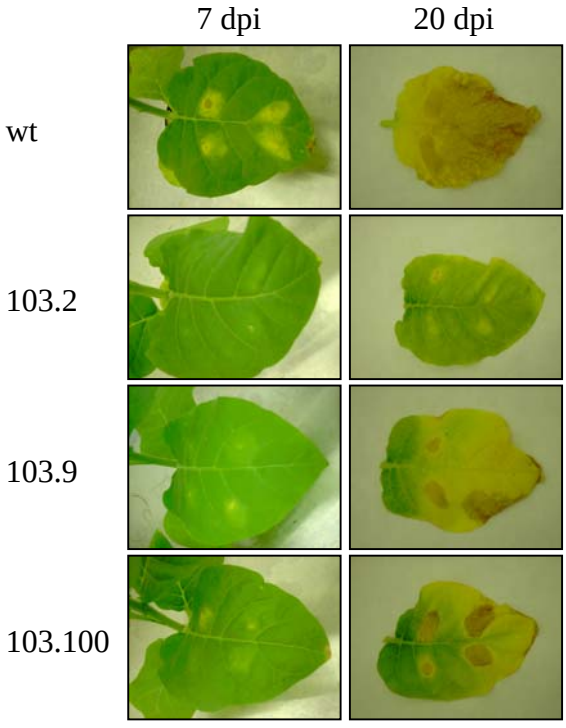

Fig. 10 *Nicotiana* wild-type and transgenic lines inoculated with *P. syringae* pv *tabaci*. Fully developed young leaves of wild-type (wt) and transgenic lines overexpressing *AtStr1* (103.2; 103.9; 103.100) were inoculated with 10^8 cfu of *P. syringae* pv *tabaci* at four injections points per leaf. Development of symptoms was visually monitored 7 (left) and 20 dpi (right).

Discussion

In the *Arabidopsis thaliana* genome 20 putative Str encoding gene sequences have been identified by database searches. In this work T-DNA insertion mutants of *AtStr1*, *AtStr2*, *AtStr11* and *AtStr15* as well as transgenic *Nicotiana tabacum* plants heterologously overexpressing *AtStr1* from *Arabidopsis* have been characterised to investigate the biological functions of the Str proteins.

To identify plants containing a T-DNA insertion in the genome, selected mutant plants were screened by PCR using primers flanking the T-DNA insertion. This has identified at least one line of *atstr1*, *atstr2*, *atstr11* and *atstr15* mutants containing the T-DNA insertion. However, the possibility of additional insertions independent of the one desired in the respective *Str* gene sequence cannot be excluded from the PCR results. We finally confirmed that two T-DNA insertion mutant lines of *atstr1* gave a single signal in Southern blot analysis using gene specific probes generated against *NTPII* indicating the interruption of the *AtStr1* gene in this mutant. Although the *atstr15* mutants were shown to carry a second independent insertion in their genome by Southern blot analysis and thus, a second independent gene of unknown identity might be knocked out as well and although in most analysed mutant lines of *atstr2* and *atstr11* no insertion could be detected by PCR or Southern blot analysis, experiments have been conducted with these plants. The *AtStr15* gene was believed to be interrupted in *atstr15* lines, and *atstr2* and *atstr11* displayed altered phenotypes compared to the wild-type.

Str activity was determined in total protein extracts from *Arabidopsis* wild-type and transgenic insertion lines using 3-MP or TS as sulfur donor substrate. Both substrates used in the *in vitro* enzyme activity assays could be metabolised by Str *in vivo*. In mammals 3-MP is produced by transamination of L-Cys by L-Cys aminotransferase or by conversion of D-Cys by D-amino acid oxidase in Cys catabolism (Huang et al., 1998). This may also be true in plants. 3-MP might be further converted by a 3-MP Str to give either pyruvate and elemental sulfur by reaction with itself, pyruvate and thiocyanate by transfer of sulfane sulfur to cyanide, or pyruvate and TS by consumption of sulfite. The TS may then be further metabolised by a Str to sulfite either by sulfur transfer to cyanide resulting in thiocyanate as second product of the reaction or by consumption of two molecules of reduced glutathione to give one molecule of oxidised glutathione and H_2S as secondary products to sulfite. The sulfite is then further oxidised to sulfate. To investigate a possible impairment of sulfur metabolism by the interruption of *Str* genes total contents of the sulfur metabolites Cys, glutathione and TS have been estimated in *Arabidopsis* T-DNA insertion mutants and wild-

type plant material by HPLC analysis. Furthermore, investigations on a possible *in vivo* function of *AtStr1* in pathogen defence and of *AtStr15* in MoCo biosynthesis have been conducted.

The mitochondrial AtStr1 protein, first described in 2000 (Hatzfeld & Saito, 2000; Heazlewood et al., 2004; Nakamura et al., 2000; Papenbrock & Schmidt, 2000a; Bauer et al., 2004) has already been the subject of several functional analyses: an involvement of AtStr1 in cyanide detoxification appears unlikely (Meyer et al., 2003) and the preliminary analysis of an *Arabidopsis* Str1 T-DNA insertion mutant suggested that AtStr1 is not directly involved in iron-sulfur cluster assembly (Nakamura et al., 2000). Still, an involvement of other Str proteins in these processes cannot be excluded. Furthermore, it has to be kept in mind that today a number of as many as 20 putative Str protein encoding genes has been found in the *Arabidopsis* genome and that another Str may have taken over the function of the knocked out *AtStr1* gene, e.g. AtStr2.

The interruption of *AtStr1* did not produce a phenotype distinct from the wild-type in *Arabidopsis* mutants. However, since the *atstr1* mutants displayed an enhanced kanamycin sensitivity only very small amounts of plant material were available so that *atstr1* mutants could not be included in the enzyme activity assays and estimation of sulfur metabolite contents. Transgenic lines may show silencing of the *NPTII* kanamycin resistance gene after several generations of growth. Thus, it is not unusual for a mutant line to not express the drug resistance phenotype. To further investigate a possible involvement of Str in plant defence against phytopathogens, transgenic *Nicotiana* lines overexpressing *AtStr1* from *Arabidopsis* and wild-type plants have been inoculated with *Pseudomonas syringae* pv *tabaci*, the causal agent of tobacco wildfire (bacterial leaf blight). The *Nicotiana* mutants have been verified by PCR and Western blot analysis using monospecific antibodies raised against AtStr1. Total Str activity was increased in the transgenic lines compared to wild-type. The abundance of AtStr1 and total Str activity were higher in older than in younger leaves of the mutants. The results from a first experiment are very promising: in the transgenic *Nicotiana* lines infected with *P. syringae* chlorosis and necrosis symptoms have been less severe than in infected wild-type plants. Thus, AtStr1 may play a role in plant protection, in pathogen defence reactions or in general stress response to various abiotic and biotic triggers. Further experiments need to be done to confirm these hypotheses. Rhd proteins have been proposed to detoxify reactive oxygen species in mammals (Nandi et al., 2000); it might be speculated about a possible protective function of the AtStr proteins during pathogen defence in protecting healthy tissue from oxidative damage.

Since the abundance of *AtStr2* mRNA and total Str activity using 3-MP as sulfur donor substrate were shown to increase with age in *Arabidopsis* plants a role of the cytoplasmic AtStr2 as proposed also for AtStr1 in mobilising sulfane-sulfur during senescence for transport processes was suggested (Bauer et al., 2004; Hatzfeld & Saito, 2000; Heazlewood et al., 2004; Nakamura et al., 2000; Papenbrock & Schmidt, 2000b). The interruption of the *AtStr2* gene produced a strong phenotype of misshaped first leaves and asymmetric rosettes in juvenile plants. However, after several weeks of growth no difference to the wild-type was observed. A reduction of Str activity could be reported in *Arabidosis atstr2* mutant lines using 3-MP as sulfur donor. The protein encoded by the interrupted *AtStr2* gene has been shown to prefer 3-MP over TS as sulfur donor substrate *in vitro* in previous studies (Papenbrock & Schmidt, 2000b). In *atstr2* line 2.B.5 the glutathione and TS content were significantly reduced, though. Since there was no difference in total Cys content in this mutant, a step in the metabolic pathway leading from Cys to glutathione may be impaired. However, another metabolic pathway or a signal cascade must be affected by the interruption of the *AtStr2* gene leading to the drastic deformations of first leaves in the juvenile developmental state of the mutant plants.

AtStr11 contains only an inactive Rhd domain, lacking the catalytically important Cys residue in the active-site loop that binds the sulfane sulfur to be transferred in a persulfide bond during the reaction. A functional role of this protein in sulfur transfer was thus questioned. Therefore the strong alterations found in the mutant plants were not expected. The strong stunted phenotype of *atstr11* mutants and results from enzyme activity assays showing reduced Str activity using 3-MP as sulfur donor substrate and from HPLC analysis revealing a reduction in total TS content compared to the wild-type indicate that the protein does indeed fulfil a role in sulfur metabolism. AtStr11 may have a supporting function in enhancing or maintaining 3-MP Str activity of other Str, maybe by stabilising the protein or a reaction intermediate during catalysis. Or it may act in signalling or regulation of the activity of other Str enzymes. The protein has not been the subject of any other investigations described in the literature. Up to now no protein consisting of only the inactive Rhd domain has been described. However, it has to be kept in mind that the mutants have not been properly verified yet. This is especially important in the case of AtStr11 since several mutant lines show a strong stunted phenotype even under standard growth conditions but the *NPTII* insertion could only be detected in one of the mutant lines by PCR. Further investigations need to be done to reveal the functional role of AtStr11 in the plant when the verification of the mutants has been completed.

The chloroplast thylakoid membrane protein AtStr15 (SEN1) (Bauer et al., 2004) shows high sequence similarity to the senescence-associated, dark-inducible proteins Ntdin and Rsdin1 from tobacco and radish, respectively (Oh et al., 1996; Shimada et al., 1998; Yang et al., 2003) and further displays similarity to small stress-related proteins such as sulfide dehydrogenase (sud) from *Wolinella succinogenes*, catalysing sulfide oxidation and the *E. coli* GlpE and PspE proteins. Based on sequence motif similarities, a regulation of the expression by various stresses, heat shock and phytohormones has been suggested as well as a repression of the promotor by sugars has been shown (Oh et al., 1996; Chung et al., 1997; Fujiki et al., 2000). Since contradictory results were obtained in experiments using leaf material or whole plants, the effects of absisic acid and ethylene, both important hormonal factors in promoting senescence of plant organs, are not clear (Chung et al., 1997; Oh et al., 1996; Schenk et al., 2005). It has been speculated that the protein may be involved in a certain cellular redox reaction (Oh et al., 1996). Very recently, an up-regulation of the expression in response to compatible as well as incompatible fungal pathogens and virulent and avirulent bacterial pathogens was shown (Schenk et al., 2005).

The interruption of *AtStr15* did not produce a phenotype different to the wild-type in the investigations performed in this work. Alterations neither in habitus nor in Str activity using 3-MP or TS as sulfur donor substrate nor in sulfur metabolite content could be observed in *atstr15* mutants compared to wild-type plants. Also in another very recent work analysing *atstr15* mutants a phenotype distinguishable from the wild-type phenotype could be observed neither under normal or high light conditions nor after infection with the fungal pathogen *Fusarium oxysporium* (Schenk et al., 2005). Due to the presence of multiple isoforms within Str group VI a phenotype may be masked by a take-over of the function of the knocked out AtStr15 by another Str and detectable only at very specific conditions. Also other investigators were faced with this problem when trying to characterise knock-out mutants of single Str genes in bacteria. The interruption of a gene did not produce a phenotype under several conditions (Colnaghi et al., 1996). The large number of similar enzymes, their differential and/or simultaneous expression, leading to possible synergistic and antagonistic effects make the characterisation very labour-intensive. One may have to extensively vary growth and environmental conditions to be sure whether *atstr15* Str knock-out mutants produce a phenotype or not. For this reason it will be advantageous to analyse double mutants with two or even more of the putative Str enzymes of one group knocked out. Another alternative to investigate the biological role of closely related proteins is RNA interference. In the genome of the cyanobacterium *Synechococcus elongatus* PCC 7942 so

far only three putative Str genes (moeB, rhdA, sex3034) have been identified compared to 20 putative Str genes in *Arabidopsis*. Hence, it will be much easier to produce a total knock-out mutant of Str activity in this organism than in *Arabidopsis*. However, the transformation of the cyanobacterium with the respective constructs has not been successful in our hands thus far. Valuable clues are expected from these experiments for further investigations on the physiological function(s) of Str proteins in *Arabidopsis*.

Recently, Ntdin from tobacco displaying 56.8% sequence homology to AtStr15, was shown to be involved in MoCo biosynthesis via a chlorate sensitivity test (Yang et al., 2003). MoCo-deficient plant mutants show a pleiotropic loss of all four molybdenum-enzyme activities, nitrate reductase, xanthine dehydrogenase, aldehyde oxidase and sulfite oxidase (reviewed in Mendel & Haensch, 2002). Plants impaired in MoCo biosynthesis will not be able to metabolise the toxic nitrate reductase substrate analogue and grow on medium containing chlorate without impairment. After 30 days of growth on chlorate supplemented medium, there was no difference observable between *atstr15* mutant lines and the wild-type. An involvement of AtStr15 in MoCo biosynthesis as shown for Ntdin in tobacco thus appears rather unlikely. Very recent results of another group confirm this finding (Schenk et al., 2005). A MoeB (molybdopterin synthase sulphurylase) motif present in the Ntdin protein sequence and several *Arabidopsis* proteins involved in MoCo synthesis like Cnx proteins can not be found in the AtStr15 sequence (Schenk et al., 2005). Thus, despite the high sequence similarity to Ntdin a proposed function of AtStr15 in MoCo biosynthesis can be excluded. The *Arabidopsis* protein fulfils a distinct yet unknown biological function in the plant.

Stronger alterations in sulfur metabolite contents were presumed since the plants in which *AtStr2* or *AtStr11* were interrupted showed phenotypes obviously different to the wild-type phenotype even under standard growth conditions. Still, alterations especially of Cys content could not necessarily be expected since the Cys pool is filled and used by different metabolic pathways and the Cys content is tightly regulated (Rausch & Wachter, 2005). Therefore, significant changes can not be expected from the interruption of one single gene, especially when there are numerous isoforms of the encoded protein present in the plant that may take over the function(s) of the interrupted protein and compensate this loss.

Using TS as sulfur donor substrate no significant differences in total Str activity could be observed between wild-type and *atstr2, atstr11* and *atstr15* transgenic lines. A reason for the fact that there are no differences in the lines analysed could be that on one hand only two sulfur donor substrates (3-MP, TS) and one sulfur acceptor (KCN) were tested. Both substrates used in the *in vitro* enzyme activity assays could be metabolised naturally. Still,

the physiological levels of 3-MP are rather low in comparison to the K_m determined *in vitro* (Papenbrock & Schmidt, 2000b). The kinetic data may therefore indicate that better substrates still need to be found (Burow et al., 2002) and the *in vivo* substrate(s) of the enzymes may be others than those used in these assays. Besides further sulfur containing substrates, compounds containing phosphorus or the chemically similar arsenic could be candidates since Rhd domains are structurally related to the catalytic subunit of Cdc25 phosphatases (Bordo & Bork, 2002; Forlani et al., 2003; Hofmann et al., 1998). Indeed AtStr5 has just recently been shown to be a tyrosine phosphatase (Landrieu et al., 2004). Furthermore, a mammalian recombinant selenium-bound Rhd was shown to serve as selenium donor in an *in vitro* selenophosphate synthetase assay and might act in the synthesis of selenoproteins (Ogasawara et al., 2001). Thus, some of the putative Str proteins in *Arabidopsis* may not be involved in sulfur but in phosphorus, arsenic or selenium metabolism. First investigations on phosphatase activity of AtStr have been performed. However, for the six proteins tested so far, no phosphatase activity could be determined (Hartmann & Papenbrock, unpublished data).

The *AtStr15* homologue *Ntdin* from tobacco also has similarity to *AtStr17* (Yang et al., 2003) which is suggested to be a pseudogene since no expressed source tags are found for this protein (Bauer et al., 2004). Furthermore, AtStr13 is annotated in databases as molybdopterin synthase sulphurylase and was recently suggested to be involved in auxin-signalling in *Arabidopsis* (Zhao et al., 2003). When knock-out mutants of *AtStr13* are available, an involvement in MoCo biosynthesis of these proteins should be investigated in the chlorate sensitivity test.

Further investigations need to be done to clarify the functional role of AtStr in the plant.

CHAPTER VI

General discussion

Group I AtStr in a global Str context, focusing on structure and possible *in vivo* functions

In *Arabidopsis* sulfurtransferase (Str) proteins were found as single- or two-domain proteins with the C-terminal domain hosting the catalytic cysteine (Cys) residue or composed of a rhodanese (Rhd) domain in combination with distinct protein domains. AtStr1 (At1g79230) and AtStr2 (At1g16460) in group I are two-domain Str proteins localized in mitochondria and the cytoplasm, respectively. The proteins show 81% homology to each other on the amino acid level and resemble the 3-mercaptopyruvate (3-MP) Str from mammals (Hatzfeld & Saito, 2000; Heazlewood et al., 2004; Nakamura et al., 2000; Papenbrock & Schmidt, 2000a, b; Bauer et al., 2004). Apart from *Arabidopsis*, sequences of plant two-domain Str were also described in *Datisca glomerata* (Okubara & Berry, 1999) and wheat (Niu et al., 2002). Crystal structures of the two-domain bovine Rhd (Ploegman et al., 1978) and *Azotobacter vinelandii* RhdA (Bordo et al., 2000) showed that both thiosulfate (TS) Str enzymes display very similar three-dimensional conformations being composed of two identically folded globular domains connected by an interdomain linker sequence (Bordo & Bork, 2002). Recently, two-domain 3-MP Str from *Leishmania major* (Alphey et al., 2003) and *Escherichia coli* (SseA) (Spallarossa et al., 2004) were crystallized. Both enzymes display specific structural differences relative to eukaryotic and prokaryotic two-domain TS Str concerning the conformation of the active-site loop. Besides many similarities, such as the conserved Cys residue, the characterised two-domain plant Str differ in their structure from the two-domain mammalian TS and 3-MP Str. The number and positions of Cys residues, and consequently maybe also the function in plant Str, are different from known Str sequences in other organisms (Burow et al., 2002). In the AtStr1C332S mutant the catalytic Cys was substituted by serine. In spectroscopic analyses performed in this thesis it could be shown that the previously reported loss of Str activity in this mutant (Burow et al., 2002) is due not only to the loss of the persulfuration site but also to conformational changes of the whole protein structure. Of the five Cys residues in AtStr1 a second Cys (C339) close to the catalytic C332 was suggested to be involved in catalysis. The Str activity of the AtStr1C339V mutant was shown to be reduced to 25% using TS as sulfur donor substrate and slightly but

significantly using 3-MP (Burow et al., 2002). The substitution of C339 by valine did not alter the conformation of the protein as shown by spectroscopic analyses performed in this work (Chapter II, Bartels et al., submitted), thus supporting a catalytic rather than a structural role of C339. The Cys residue might act in recognising and binding of the acceptor molecule in close vicinity to the active-site.

The interdomain linker connecting both domains may have a role in positioning of the two domains to each other to provide an appropriate conformation for substrate binding. In the *E. coli* two-domain RNA polymerase, deletion, insertion or substitution of one to ten amino acids in the interdomain linker clearly affected the activity (Fujita et al., 2000). By mutagenic insertion of two amino acids in the interdomain tether of bovine Rhd the activity of the protein was only slightly decreased but stability was considerably reduced. The linker was thus suggested to be of importance for stability and conformation of the protein (Luo et al., 1995). In a previous study the interdomain linker in the two-domain AtStr1 protein was shown to play a role in stabilising the protein and maintaining the activity in the presence of urea and at increased temperature (39 and 41°C) (Burow et al., 2002). In this work, the prolonged linker sequence in AtStr1 was confirmed to be important in maintaining the correct conformation and stability of the protein and thereby also the enzyme activity. The AtStr1wlink mutant, deleted of the plant specific elongation of the linker sequence was demonstrated to be far more prone to proteolytic digestion by trypsin than the wild-type AtStr1 (Chapter II, Bartels et al., submitted). The plant specific elongation of the interdomain linker sequence in AtStr1 was suggested to provide an extended hydrophobic environment surrounding the substrate binding site, enabling the protein to bind substrates as large as proteins. If this is true, the plant two-domain Str might act in the regulation of other proteins by the direct transfer of sulfane sulfur (Burow et al., 2002).

Further experiments performed in this work aimed at the elucidation of the proteins' function *in vivo*. In studies on the regulation of gene expression under various environmental conditions, the expression of group I genes was not significantly affected by different nutritional conditions or light/dark cycling but increased with age (Chapter IV, Bartels et al., submitted). A role of AtStr1 and AtStr2 in the mobilisation of sulfur-metabolites from senescing tissues for the transport to younger tissues has been proposed (Papenbrock & Schmidt, 2000b). The expression levels of developmentally regulated AtStr are also increased by the senescence-promoting phytohormone ethylene or its precursor

1-aminocyclopropane-1-carboxylic acid (Meyer et al., 2003; data not shown), supporting a role of the proteins in senescence.

Transgenic *Nicotiana* plants overexpressing *AtStr1* showed less severe chlorosis and necrosis symptoms after infection with *Pseudomonas syringae* pv *tabaci* than wild-type plants in first preliminary experiments. AtStr1 is thus suggested to be involved in plant protection, pathogen defence reactions or in a general stress response of plants. Since Rhd proteins have been proposed to detoxify reactive oxygen species in mammals (Nandi et al., 2000) one could speculate about a possible protective function of the AtStr proteins during pathogen defence by protecting healthy tissue from oxidative damage.

In contrast to an unaltered phenotype in *atstr1* T-DNA insertion mutants, the interruption of *AtStr2* caused a strong phenotype of misshaped first leaves. AtStr2 hence might have a regulatory impact on certain pathways by signalling or activation / deactivation of other proteins leading to this malformation (Chapter V).

Group IV AtStr in a global Str context, focusing on subcellular localisation and possible *in vivo* functions

For the three proteins, AtStr9 (At2g42220), AtStr10 (At3g08920) and AtStr11 (At4g24750), of group IV no putative function or homology to other proteins has been annotated in the databases. In this work the three single-domain proteins have been characterised (Chapter III). While AtStr9 and AtStr10 are likely to be active enzymes, AtStr11 is believed to be inactive since in this protein the catalytic Cys residue is replaced by alanine. A persulfide formation with the substrate as observed for bovine Rhd (Westley, 1973) thus can be excluded. Inactive Rhd domains have been suggested to play a role in regulation and/or signalling in combination with distinct protein domains (Bordo & Bork, 2002). Thus far, no protein consisting of only the inactive Rhd domain has been described.

Since the proteins were expressed in an insoluble state in *E. coli*, the proteins could not be analysed on the catalytic level so far. Substrate specificity and kinetic properties therefore remain to be elucidated. For the evaluation of the biological function of a protein the knowledge of its subcellular localisation might provide valuable information. Especially in plants compartmentation plays an important role in regulation and communication of cellular processes (Papenbrock & Grimm, 2001). Str have been identified in different compartments in the organisms analysed so far. Seven Str proteins were identified in *E. coli*. The single-domain GlpE protein, showing the highest similarity to the single-domain AtStr in group VI, was localised in the cytoplasm while at least one two-domain Str was found in the periplasm (Ray et al., 2000). In the cyanobacterium

Synechococcus sp. strain PCC 7942 a two-domain Rhd-like protein was found in the periplasmic space and was suggested to be involved in transport processes of specific sulfur compounds (Laudenbach et al., 1991). Two distinct two-domain Str proteins have been identified in mammalia displaying 3-MP and TS specific Str activity, respectively. The 3-MP Str was localised in the cytoplasm and mitochondria by immunogold-labelling and Western blot analysis, while the TS Str was exclusively found in mitochondria, mainly in liver cells. The 3-MP Str might detoxify cyanide in the cytoplasm while in mitochondria both Str may effectively protect cytochrome c oxidase (Nagahara et al., 1995, 1999). So far, two-domain Str of group I and Str containing one Rhd domain of groups II, IV and VI in *Arabidopsis* have been detected in the chloroplast, the mitochondrion and the cytoplasm using different methods (Bauer et al., 2004; Hatzfeld & Saito, 2000; Heazlewood et al., 2004; Nakamura et al., 2000; Papenbrock & Schmidt, 2000a; Peltier et al., 2004). However, the data available on Str in general indicate that it has to be discriminated between single- and two-domain Str. In this work AtStr9 was localised in chloroplasts and AtStr10 in mitochondria by transient transformation of fusion constructs with the green fluorescent protein (GFP) in *Arabidopsis* protoplasts. Computer-based predictions indicate a possible membrane association of the C-terminal sequence of both proteins, explaining the insolubility of the proteins after expression in *E. coli*. This is supported by some similarity of the distribution pattern inside the cell of the fluorescent GFP constructs of AtStr9 and AtStr10 and the thylakoid membrane protein AtStr15 (At4g35770) (Bauer et al., 2004). AtStr9 has recently been identified in the proteome of the thylakoid membrane by three-phase-partitioning and mass spectrometry. In this approach also AtStr4 (At4g01050), AtStr4a (At3g25480) and AtStr14 (At4g27700) have been shown to be thylakoid membrane localised (Peltier et al., 2004). Further the expression of AtStr9 was shown to be clearly light induced pointing to a possible role of the thylakoid membrane protein in photosynthesis or some pathway closely connected to photosynthesis. In cadmium-tolerant *Datura innoxia* the expression of a homologue of AtStr9 was increased by cadmium as shown in a suppressive subtractive hybridization approach used to create a library enriched in cadmium-induced cDNAs. The coupling of a TS reductase to the Cys biosynthetic pathway by a Str may help to provide sulfide for Cys biosynthesis independent of the regular sulfate assimilatory pathway to meet the increased demand for glutathione and phytochelatins during heavy metal stress (Louie et al., 2003). While the expression of *AtStr9* and *AtStr10* was unimpaired by age both proteins displayed a cycling in transcript level in the diurnal light/dark cycle, though the expression pattern revealed a

distinct rhythm of regulation than the applied light/dark rhythm. A circadian regulation of the expression of these genes should be checked in continuous light or dark conditions.

Since AtStr11 lacks the catalytic Cys residue in the active-site a functional role of this protein in sulfur metabolism was questioned. Accordingly, the expression of *AtStr11* was not altered by sulfate, TS or phosphate nutrition. In contrast to most other *AtStr* genes the expression of group IV genes did not increase with age. Interestingly, *atstr11* T-DNA insertion mutants displayed a strong stunted phenotype. Since the 3-MP Str activity in total protein extracts and also total TS content in the mutant plants were reduced, AtStr11 is believed to indeed fulfil a role in sulfur metabolism. The protein may have a supporting function in enhancing or maintaining 3-MP Str activity of other Str for example by stabilising the protein or a reaction intermediate during catalysis. Or it may act in signalling or regulation of the activity of other Str enzymes.

Group VI AtStr in a global Str context, focusing on possible *in vivo* functions

Most group VI AtStr have been annotated in the databases as senescence-associated proteins. The proteins share similarity to the C-terminal domain of AtStr1 and to the GlpE protein in *E. coli* (Ray et al., 2000) as well as to other distinct stress-related proteins such as sulfide dehydrogenase from *Wolinella succinogenes* (Klimmek et al., 1998) and phage-shock protein PspE from *E. coli* (Adams et al., 2002). While AtStr14, AtStr15 and AtStr16 are located in the chloroplasts, AtStr18 was found in the cytplasm (Bauer et al., 2004).
In agreement with the database annotations the expression of *AtStr15*, *AtStr16* and *AtStr18* as well as Str activity in total protein extracts have been shown to increase with progressing age in this work. In contrast, the expression of *AtStr14* decreased in five-week-old *Arabidopsis* plants and also at low sulfate supply, indicating a distinct role in sulfur metabolism. While the 3-MP Str activity in total protein extracts increased continually with progressing age of the plants, the TS Str activity was strongly induced from week five to the end of the experiment after six weeks. The expression patterns of *AtStr16* and *AtStr18* as well as that of the senescence-associated *SAG13* used as a marker for senescence in a previous work (Meyer et al., 2003) paralleled with the pattern of TS Str activity in this work (Chapter IV, Bartels et al., submitted). AtStr16 (At5g66040) and AtStr18 (At5g66170) have been identified as TS Str proteins (Bauer et al., 2002) and are likely to play a role in senescence.

The most interesting and by far best characterised of the six proteins in group VI so far is AtStr15 (AtSEN1). The senescence-associated *SEN1* gene is induced commonly during

senescence caused by several senescence inducing factors including age, darkness, and phytohormones as well as by inoculation with diverse pathogens (Chung et al., 1997; Oh et al., 1996; Schenk et al., 2005; Weaver et al., 1998). In the experiments conducted in this work, *AtStr15* expression was clearly induced at all three nutritional stress conditions (low sulfate or phosphate, added TS) and in darkness and increased with age (Chapter IV, Bartels et al., submitted). In contrast to the results obtained in this work, the expression of *AtStr15* was slightly reduced in leaves and roots at low phosphate in a recent microarray analysis (Misson et al., 2005). Despite all these information, the biological function of the *AtStr15* gene product could not be determined, yet. In tobacco, a homologue of AtStr15, Ntdin, was recently shown to be involved in molybdenum cofactor (MoCo) biosynthesis (Yang et al., 2003). According to results obtained in this work, a role of AtStr15 in MoCo biosynthesis could be excluded (Chapter V). This was confirmed by very recent results from another study on the function of AtStr15/SEN1 (Schenk et al., 2005). AtStr13 (At5g55130) in group V is annotated as molybdopterin synthase sulphurylase in databases. The AtStr13/SIR1 protein is composed of a Rhd-like domain and a ubiquitin-activating enzyme E1-like domain. SIR1 is a regulator of many auxin-inducible genes. The *sir1* mutant was resistant to sirtinol, a small molecule activating many auxin-inducible genes, and displays auxin-related developmental phenotypes. An involvement of the protein in the propagation of auxin-signals in *Arabidopsis* was suggested (Zhao et al., 2003). Knock-out mutants of *AtStr13* should be analysed concerning an involvement of the gene product in MoCo biosynthesis. Taking together the information obtained on AtStr15/SEN1 thus far, the protein may have a function in general stress response of the plant caused by various biotic and abiotic triggers.

General aspects on substrate specificity and conclusions

Since Str / Rhd proteins are widely distributed among eukaryotes and prokaryotes an involvement in essential metabolic pathways seems likely. However, still little is known about the true biological functions of the proteins *in vivo*.

In silico studies revealed that Rhd domains are structurally related to the catalytic subunit of Cdc25 phosphatase and arsenate reductase enzymes (Hofmann et al., 1998). The proteins are likely to share a common evolutionary ancestor (Bordo & Bork, 2002). Furthermore, Rhd-like enzymes in *E. coli*, *Leishmania*, *Azotobacter* and bovine have been shown to be able to bind selenium instead of sulfur at the catalytic Cys *in vitro* and to transfer it to an acceptor molecule (Melino et al., 2003; Ogasawara et al., 2001, 2004; Ray

et al., 2000; Williams et al., 2003). The size and amino acid composition of the catalytic loop of the protein are playing a key role in substrate recognition and specificity (Bordo & Bork, 2002). The mutagenic elongation of the active-site loop of *A. vinelandii* RhdA by one additional amino acid changed the selectivity of the enzyme from sulfur to phosphorus containing substrates (Forlani et al., 2003). Thus supporting the hypothesis that Rhd domains displaying a seven-amino-acid active-site loop are able to bind substrates containing phosphorus or the chemically similar arsenic, whereas Rhd-like domains displaying a six-amino-acid loop with Cys at the first position interact with substrates containing reactive sulfur or selenium (Bordo & Bork, 2002). The putative AtStr proteins thus may not all metabolise substrates containing sulfur but also substrates containing phosphorus, arsenic or selenium.

Two types of Str have been described in *Arabidopsis* concerning the sulfur donor substrate used (*in vitro*) – two two-domain 3-MP Str and two single-domain TS Str (Bauer et al., 2002, 2004; Hatzfeld & Saito, 2000; Nakamura et al., 2000; Papenbrock & Schmidt, 2000a, b). In this work, the impact of the nutritional supply of *Arabidopsis* on the expression and activity of Str was investigated (Chapter IV, Bartels et al., submitted). The concentration of sulfate as the main sulfur source of plants, TS as substrate of the TS Str reaction and phosphate as possible substrate was altered in controlled feeding experiments. The increase of TS Str activity observed at the low sulfate concentration indicates an impairment of the metabolism by shortened sulfate supply as could be expected. The TS available might be degraded by Str to thiocyanate and sulfite, and sulfite reassimilated to Cys to fill up the Cys pool for further biosynthetic pathways. Since neither expression of the AtStr investigated nor total Str activity were significantly affected by the high concentration of TS the translation products of constitutively expressed Str might be sufficient for the catabolism of additional TS. Since 3-MP Str activity was induced at the low phosphate concentration a role of at least some of the putative Str in phosphate metabolism appears likely. Very recently, the putative AtStr5 protein has been identified as tyrosine phosphatase being able to use *p*-nitrophenyl phosphate as substrate and to stimulate the kinase activity of *Arabidopsis* cyclin dependent kinases *in vitro* (Landrieu et al., 2004). First investigations on phosphatase activity of AtStr proteins AtStr1, AtStr2, AtStr7 (At2g40760), AtStr14, AtStr16, and AtStr18 using 3-*o*-methylfluorescein-phosphate as substrate did not reveal any phosphatase activity under the conditions applied (Hartmann & Papenbrock, unpublished data). Duan et al. (2005) indirectly demonstrated

arsenate reductase activity of the same protein (AtStr5). The arsenate reductase activity present in wild-type plants was lost in *Arabidopsis atStr5* knock-out mutants.

However, the *in vivo* substrates of most members of the Str / Rhd multi protein family have not been identified. Since the 20 putative AtStr unified in the multi protein family by one or more Rhd domains display very high diversity among each other, each single protein needs to be individually analysed. In fact the more experimental data are collected about the putative AtStr and Str / Rhd proteins in general the more obvious becomes the vast heterogeneity of this multi protein family. Large scale enzyme activity assays for testing numerous different substrates of the putative AtStr are labour intensive and time consuming. The purification of large amounts of recombinant proteins will be needed for a first determination of the most probable substrates *in vitro.* Crystallisation studies on the structure of the AtStr proteins may reveal the properties of the active-site loop and help to classify the proteins. So far, the three-dimensional structure has been resolved only for the non-catalytic Rhd homology domain of AtStr4 (At4g01050) (Pantoja-Uceda et al., 2005).

The large number of putative AtStr proteins that are differentially and/or simultaneously expressed, leads to possible synergistic and antagonistic effects. The AtStr proteins are likely to be active in different compartments at different time points during the day and there probably exists some redundancy in function of the proteins. This might explain why there are so little differences in the specific enzyme activities determined in total protein extracts. Therefore double or triple knock-out or RNAi mutants need to be generated to identify the *in vivo* functions of the Str in plants.

Overexpression of the respective proteins in a soluble state will allow the detailed biochemical analysis of the catalytic functions, and together with *in planta* studies using transgenic plants, will enable the regulation of the expression of the encoding genes and roles in specific pathways to be elucidated.

Furthermore, the collection of expression patterns of Str proteins from microarray analyses may be a promising approach to clarify the functional role of the distinct AtStr proteins in the plant organism. This could be a helpful tool especially in the case of this very divergent multi protein family.

From the experimental data available, an involvement of Str in biotic and abiotic stress response reactions of plants appears likely. However, the mutants analysed in this work still have to be properly verified. Taken together the results obtained from the distinct analyses of AtStr in this work may provide a good basis for further investigations.

REFERENCES

AGRIOS GN (1997) Plant diseases caused by prokaryotes: bacteria and mollicutes. Chapter 12 in Plant pathology 4th ed, Harcourt/Academic Press, Burlington, Massachusettes, USA. pp 407-470

ADLER AJ, GREENFIELD NJ, FASMAN GD (1973) Circular dichroism and optical rotatory dispersion of proteins and polypeptides. Methods Enzymol 27*:* 675-735

ALONSO JM, STEPANOVA AN, LEISSE TJ, KIM CJ, CHEN H, SHINN P, STEVENSON DK, ZIMMERMAN J, BARAJAS P, CHEUK R, GADRINAB C, HELLER C, JESKE A, KOESEMA E, MEYERS CC, PARKER H, PREDNIS L, ANSARI Y, CHOY N, DEEN H, GERALT M, HAZARI N, HOM E, KARNES M, MULHOLLAND C, NDUBAKU R, SCHMIDT I, GUZMAN P, AGUILAR-HENONIN L, SCHMID M, WEIGEL D, CARTER DE, MARCHAND T, RISSEEUW E, BROGDEN D, ZEKO A, CROSBY WL, BERRY CC, ECKER JR (2003) Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science 301: 653-657

AZUMI Y & WATANABE A (1990) Evidence for a senescence-associated gene induced by darkness. Plant Physiol 95: 577-583

BABIYCHUK E, KUSHNIR S, BELLES-BOIX E, VAN MONTAGU M, INZE D (1995) *Arabidopsis thaliana* NADPH oxidoreductase homologs confer tolerance of yeasts toward the thiol-oxidizing drug diamide. J Biol Chem 270: 26224-26231

BAUER M, DIETRICH C, NOWAK K, SIERRALTA WD, PAPENBROCK J (2004) Intracellular localization of Arabidopsis sulfurtransferases. Plant Physiol 135: 916-926

BAUER M & PAPENBROCK J (2002) Identification and characterization of single-domain thiosulfate sulfurtransferases from *Arabidopsis thaliana*. FEBS Lett 532: 427-431

BONOMI F, PAGANI S, CERLETTI P, CANNELLA C (1977) Rhodanese-mediated sulfur transfer to succinate dehydrogenase. Eur J Biochem 72: 17-24

BORDO D & BORK P (2002) The rhodanese/Cdc25 phosphatase superfamily: Sequence-structure-function relations. EMBO Rep 3: 741-746

BORDO D, DERIU D, COLNAGHI R, CARPEN A, PAGANI S, BOLOGNESI M (2000) The crystal structure of a sulfurtransferase from *Azotobacter vinelandii* highlights the evolutionary relationship between the rhodanese and phosphatase enzyme families. J Mol Biol 298: 691-704

BRADFORD MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254

BUHOT C, CHENAL A, SANSON A, POUVELLE-MORATILLE S, GELB MH, MENEZ A, GILLET D, MAILLERE B (2004) Alteration of the tertiary structure of the major bee venom allergen Api m 1 by multiple mutations is concomitant with low IgE reactivity. Protein Sci 13: 2970-2978

BUROW M, KESSLER D, PAPENBROCK J (2002) Enzymatic activity of the *Arabidopsis* sulfurtransferase resides in the C-terminal domain but is boosted by the N-terminal domain and the linker peptide in the full-length enzyme. Biol Chem 383: 1363-1372

CANNELLA C, BERNI R, ROSATO N, FINAZZI AGRO A (1986) Active site modifications quench intrinsic fluorescence of rhodanese by different mechanisms. Biochemistry. 25*:* 7319-7323

CERLETTI P (1986) Seeking a better job for an under-employed enzyme: rhodanese. Trends Biochem Sci 11: 369-372

CHEW NY (1973) Rhodanese in higher plants. Phytochemistry 12: 2365-2367

CHOMCZYNSKI P & SACCHI N (1987) Single step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156-159

CHUNG B-C, LEE SY, OH SA, RHEW TH, NAM HG, LEE C-H (1997) The promotor activity of *sen1*, a senescence-associated gene of *Arabidopsis*, is repressed by sugars. J Plant Physiol 151: 339-345

COLNAGHI R, CASSINELLI G, DRUMMOND M, FORLANI F, PAGANI S (2001) Properties of the *Escherichia coli* rhodanese-like protein SseA: contribution of the active-site residue Ser240 to sulfur donor recognition. FEBS Lett 500: 153-156

COLNAGHI R, PAGANI S, KENNEDY C, DRUMMOND M (1996) Cloning, sequence analysis and overexpression of the rhodanese gene of *Azotobacter vinelandii*. Eur J Biochem 236: 240-248

COOPER RM, RESENDE ML, FLOOD J, ROWAN MG, BEALE MH, POTTER U (1996) Detection and cellular localization of elemental sulphur in disease-resistant genotypes of *Theobroma cacao*. Nature 379: 159-162

COOPER RM & WILLIAMS JS (2004) Elemental sulphur as an induced antifungal substance in plant defence. J Exp Bot 55: 1947-1953

DONADIO S, SHAFIEE A, HUTCHINSON R (1990) Disruption of a rhodaneselike gene results in cysteine auxotrophy in *Saccharopolyspora erythraea*. J Bacteriol 172: 350-360

DUAN GL, ZHU YG, TONG YP, CAI C, KNEER R (2005) Characterization of arsenate reductase in the extract of roots and fronds of chinese brake fern, an arsenic hyperaccumulator. Plant Physiol 138:461-469

DUBUIS P-H, MARAZZI C, STAEDLER E, MAUCH F (2005) Sulphur deficiency causes a reduction in antimicrobial potential and leads to increased disease susceptibility of oilseed rape. J Phytopathology 153: 27-36

ELLEDGE S, MULLIGAN JT, RAMER SW, SPOTTSWOOD M, DAVIES RW (1991) λYES: a multifunctional cDNA expression vector for the isolation of genes by complementation of yeast and *Escherichia coli* mutations. Proc Natl Acad Sci USA 88: 1731-1735

ESSIGMANN B, GULER S, NARANG RA, LINKE D, BENNING C (1998) Phosphate availability affects the thylakoid lipid composition and the expression of *SQD1*, a gene required for sulfolipid biosynthesis in *Arabidopsis thaliana.* Proc Natl Acad Sci USA 95: 1950-1955

FAUMAN EB, COGSWELL JP, LOVEJOY B, ROCQUE WJ, HOLMES W, MONTANA VG, PIWNICA-WORMS H, RINK MJ, SAPER MA (1998) Crystal structure of the catalytic domain of the human cell cycle control phosphatase, Cdc25A. Cell 93: 617-625

FINAZZI AGRO A, FEDERICI G, GIOVAGNOLI C, CANNELLA C, CAVALLINI D (1972) Effect of sulfur binding on rhodanese fluorescence. Eur J Biochem 28: 89-93

FORLANI F, CARPEN A, PAGANI S (2003) Evidence that elongation of the catalytic loop of the *Azotobacter vinelandii* rhodanese changed selectivity from sulfur- to phosphate-containing substrates. Prot Engineering 16: 515-519

FUJIKI Y, YOSHIKAWA Y, SATO T, INADA N, ITO M, NISHIDA I, WATANABE A (2001) Dark-inducible genes from *Arabidopsis thaliana* are associated with leaf senescence and repressed by sugars. Physiol Plant 111: 345-352

FUKAO Y, HAYASHI M, NISHIMURA M (2002) Proteomic analysis of leaf peroxisomal proteins in greening cotyledons of *Arabidopsis thaliana*. Plant Cell Physiol 43: 689-696

GARNIER J, GIBRAT JF, ROBSON B (1996) GOR method for predicting protein secondary structure from amino acid sequence. Methods Enzymol 266: 540-553

GLIUBICH F, GAZERRO M, ZANOTTI G, DELBONO S, BOMBIERI G, BERNI R (1996) Active site structural features for chemically modified forms of rhodanese. J Biol Chem 271: 21054-21061

GLIUBICH F, BERNI R, COLAPIETRO M, BARBA L, ZANOTTI G (1998) Structure of sulfur-substituted rhodanese at 1.36 Å. Res Acta Cryst D54: 481-486

HATZFELD Y & SAITO K (2000) Evidence for the existence of rhodanese (thiosulfate:cyanide sulfurtransferase) in plants: preliminary characterization of two rhodanese cDNAs from *Arabidopsis thaliana*. FEBS Lett 470: 147-150

HAUSMANN N, WERHAHN W, HUCHZERMEYER B, BRAUN HP, PAPENBROCK J (2003) How to document the purity of mitochondria prepared from green tissue of tobacco, pea and *Arabidopsis thaliana*. Phyton 43: 215-229

HEAZLEWOOD JL, TONTI-FILIPPINI JS, GOUT AM, DAY DA, WHELAN J, MILLAR AH (2004) Experimental analysis of the *Arabidopsis* mitochondrial proteome highlights signaling and regulatory components, provides assessment of targeting prediction programs, and indicates plant-specific mitochondrial proteins. Plant Cell 16: 241-25

HOEFGEN R & WILLMITZER L (1992) Biochemical and genetic analysis of different patatin isoforms expressed in various cultivars of potato. Plant Sci 66: 221-230

HOFMANN K, BUCHER P, KAJAVA AV (1998) A model of Cdc25 phosphatase catalytic domain and Cdk-interaction surface based on the presence of a rhodanese homology domain. J Mol Biol 282: 195-208

HOROWITZ P & CRISCIMAGNA NL (1983) The use of intrinsic protein fluorescence to quantitate enzyme-bound persulfide and to measure equilibria between intermediates in rhodanese catalysis. J Biol Chem 258: 7894-78946

HORSCH RB, FRY JE, HOFFMANN NL, EICHHOLTZ D, ROGERS SG, FRALEY RT (1985) A Simple and General Method for Transferring Genes into Plants. Science 227: 1229-1231

HUANG J, KHAN S, O'BRIEN PJ (1998) The glutathione dependence of inorganic sulfate formation from L- or D-cysteine in isolated rat hepatocytes. Chem Biol Interact 110: 189-202

JENSEN RG & BASSHAM JA (1966) Photosynthesis by isolated chloroplasts. Proc Natl Acad Sci USA 56: 1095-1101

KAKES P & HAKVOORT H (1992) Is there rhodanese activity in plants? Phytochemistry 31: 1501-1505

KARTHIKEYAN AS, VARADARAJAN DK, MUKATIRA UT, D'URZO MP, DAMSZ B, RAGHOTHAMA KG (2002) Regulated expression of *Arabidopsis* phosphate transporters. Plant Physiol 130: 221-233

KAUR Y, YBARRA J, HOROWITZ PM (2004) Active rhodanese lacking nonessential sulfhydryl groups has increased hydrophobic exposure not observed in wild-type enzyme. Protein J 23: 255-261

KELLY SM & PRICE NC (2000) The use of circular dichroism in the investigation of protein structure and function. Curr Protein Pept Sci 1: 349-384

LAEMMLI UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685

LANDRIEU I, DA COSTA M, DE VEYLDER L, DEWITTE F, VANDEPOELE K, HASSAN S, WIERUSZESKI J-M, CORELLOU F, FAURE J-D, VAN MONTAGU M, INZÉ D, LIPPENS G (2004) A small CDC25 dual-specificity tyrosine-phosphatase isoform in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 101: 13380-13385

LAUDENBACH DE, EHRHARDT D, GREEN L, GROSSMAN A (1991) Isolation and characterization of a sulfur-regulated gene encoding a periplasmically localized protein with sequence similarity to rhodanese. J Bacteriol 173: 2751-2760

LAUHON CT & KAMBAMPATI R (2000) The *iscS g*ene in *Escherichia coli* is required for the biosynthesis of 4-thiouridine, thiamin, and NAD. J Biol Chem 275: 20096-20103

LAYNE E (1957) Spectrophotometric and turbidimetric methods for measuring proteins. Methods Enzymol 3: 447-455

LEIMKUEHLER S & RAJAGOPALAN KV (2001) A sulfurtransferase is required in the transfer of cysteine sulfur in the *in vitro* synthesis of molybdopterin from precursor Z in *Escherichia coli*. J Biol Chem 276: 22024-22031

LOHMANN KN, GAN S, JOHN MC, AMASINO RM (1994) Molecular analysis of natural leaf senescence in *Arabidopsis thaliana*. Physiol Plant 92: 322-328

LOTTSPEICH F (1998) Proteinreinigung. In Lottspeich F, Zorbas H (eds) Bioanalytik. Spektrum Akad. Verl., Heidelberg; Berlin, pp 9-33

LOUIE M, KONDOR N, DEWITT JG (2003) Gene expression in cadmium-tolerant *Datura innoxia*: detection and characterization of cDNAs induced in response to Cd^{2+}. Plant Mol Biol 52: 81–89

LUO GX, HUA S, HOROWITZ PM (1995) Mutation in the interdomain tether influences the stability and refolding of the enzyme rhodanese. Biochim Biophys Acta 1252: 165-171

MANO J, TORII Y, HAYASHI S, TAKIMOTO K, MATSUI K, NAKAMURA K, INZE D, BABIYCHUK E, KUSHNIR S, ASADA K (2002) The NADPH:quinone oxidoreductase P1-zeta-crystallin in *Arabidopsis* catalyzes the alpha, beta-hydrogenation of 2-alkenals: detoxification of the lipid peroxide-derived reactive aldehydes. Plant Cell Physiol 43: 1445-1455

MATTHIES A, RAJAGOPALAN KV, MENDEL RR, LEIMKUEHLER S (2004) Evidence for the physiological role of a rhodanese-like protein for the biosynthesis of the molybdenum cofactor in humans. Proc Natl Acad Sci USA 101: 5946–5951

MELINO S, CICERO DO, FORLANI F, PAGANI S, PACI M (2004) The N-terminal rhodanese domain from *Azotobacter vinelandii* has a stable and folded structure independently of the C-terminal domain. FEBS Lett 577: 403-408

MENDEL RR & HAENSCH R (2002) Molybdoenzymes and molybdenum cofactor in plants. J Exp Bot 53: 1689-1698

MEYER T, BUROW M, BAUER M, PAPENBROCK J (2003) *Arabidopsis* sulfurtransferases: investigation of their function during senescence and in cyanide detoxification. Planta 217: 1-10

MILLER-MARTINI DM, CHIRGWIN JM, HOROWITZ PM (1994a) Mutations of noncatalytic sulfhydryl groups influence the stability, folding, and oxidative susceptibility of rhodanese. J Biol Chem 269: 3423-3428

MILLER-MARTINI DM, HUA S, HOROWITZ PM (1994b) Cysteine 254 can cooperate with active site cysteine 247 in reactivation of 5,5'-dithiobis(2-nitrobenzoic acid)-inactivated rhodanese as determined by site-directed mutagenesis. J Biol Chem 269: 12414-12418

MISSON J, RAGHOTHAMA KG, JAIN A, JOUHET J, BLOCK MA, BLIGNY R, ORTET P, CREFF A, SOMERVILLE S, ROLLAND N, DOUMAS P, NACRY P, HERRERRA-ESTRELLA L, NUSSAUME L, THIBAUD MC (2005) A genome-wide transcriptional analysis using *Arabidopsis thaliana* Affymetrix gene chips determined plant responses to phosphate deprivation. Proc Natl Acad Sci USA 102: 11934-11939

MURASHIGE T & SKOOG F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473-497

NAGAHARA N, ITO T, MINAMI M (1999) Mercaptopyruvate sulfurtransferase as a defense against cyanide toxication: molecular properties and mode of detoxification. Histol Histopathol 14: 1277-1286

NAGAHARA N, OKAZAKI T, NISHINO T (1995) Cytosolic mercaptopyruvate sulfurtransferase is evolutionarily related to mitochondrial rhodanese: striking similarity in active site amino acid sequence and the increase in the mercaptopyruvate sulfurtransferase activity of rhodanese by site-directed mutagenesis. J Biol Chem 270: 16230-16235

NAKAMURA T, YAMAGUCHI Y, SANO H (2000) Plant mercaptopyruvate sulfurtransferases: Molecular cloning, subcellular localization and enzymatic activities. Eur J Biochem 267: 5621-5630

NANDI DL, HOROWITZ PM, WESTLEY J (2000) Rhodanese as a thioredoxin oxidase. Int J Biochem Cell Biol 32: 465-473

NANDI DL & WESTLEY J (1998) Reduced thioredoxin as a sulfur-acceptor substrate for rhodanese. Int J Biochem Cell Biol 30: 973-977

NIU JS, YU L, MA Z-Q, CHEN P-D, LIU D-J (2002) Molecular cloning, characterization and mapping of a rhodanese like gene in wheat. Acta Genetica Sinica 29: 266-272

NOWAK K, LUNIAK N, WITT C, WUESTEFELD Y, WACHTER A, MENDEL RR, HAENSCH R (2004) Peroxisomal localization of sulfite oxidase separates it from chloroplast-based sulfur assimilation. Plant Cell Physiol 45: 1889-1894

OGASAWARA Y, LACOURCIERE G, STADTMAN TC (2001) Formation of a selenium-substituted rhodanese by reaction with selenite and glutathione: Possible role of a protein perselenide in a selenium delivery system. Proc Natl Acad Sci USA 98: 9494-9498

OH SA, LEE SY, CHUNG IK, LEE CH, NAM HG (1996) A senescence-associated gene of *Arabidopsis thaliana* is distinctively regulated during natural and artificially induced leaf senescence. Plant Mol Biol 30: 739-754

PAGANI S, BONOMI F, CERLETTI P (1984) Enzymic synthesis of the iron-sulfur cluster of spinach ferredoxin. Eur J Biochem 142: 361-366

PAGANI S, FORLANI F, CARPEN A, BORDO D, COLNAGHI R (2000) Mutagenic analysis of Thr-232 in rhodanese from *Azotobacter vinelandii* highlighted the differences of this prokaryotic enzyme from the known sulfurtransferases. FEBS Lett 472: 307-311

PALENCHAR PM, BUCK CJ, CHENG H, LARSON TJ, MUELLER EG (2000) Evidence that ThiI, an enzyme shared between thiamin and 4-thiouridine biosynthesis, may be a sulfurtransferase that proceeds through a persulfide intermediate. J Biol Chem 275: 8283-8286

PANTOJA-UCEDA D, LOPEZ-MENDEZ B, KOSHIBA S, INOUE M, KIGAWA T, TERADA T, SHIROUZU M, TANAKA A, SEKI M, SHINOZAKI K, YOKOYAMA S, GUNTERT P (2005) Solution structure of the rhodanese homology domain At4g01050(175-295) from *Arabidopsis thaliana*. Protein Sci 14: 224-230

PAPENBROCK J (2002) Recent results of the sulfurtransferase/rhodanese protein family. Research Signpost: Recent Res Devel Biochem 3: 13-31

PAPENBROCK J & GRIMM B (2001) Regulatory network of tetrapyrrole biosynthesis - studies of intracellular signalling involved in metabolic and developmental control of plastids. Planta 213: 667-81

PAPENBROCK J & SCHMIDT A (2000a) Characterization of a sulfurtransferase from *Arabidopsis thaliana*. Eur J Biochem 267: 145-154

PAPENBROCK J & SCHMIDT A (2000b) Characterization of two sulfurtransferase isozymes from *Arabidopsis thaliana*. Eur J Biochem 267: 5571-5579

PELTIER JB, YTTERBERG AJ, SUN Q, VAN WIJK KJ (2004) New functions of the thylakoid membrane proteome of Arabidopsis thaliana revealed by a simple, fast and versatile fractionation strategy. J Biol Chem 279: 49367-49383

PLOEGMAN JH, DRENT G, KALK KH, HOL WG (1978) The covalent and tertiary structure of bovine liver rhodanese. Nature 273: 124-129

PLOEGMAN JH, DRENT G, KALK KH, HOL WG (1979) The structure of bovine liver rhodanese. II. The active site in the sulfur-substituted and the sulfur-free enzyme. J Mol Biol 127: 149-162

POUVREAU L, KROFF T, GRUPPEN H, VAN KONINGSVELD G, VAN DEN BROEK LAM, VORAGEN AGJ (2005) Structure and stability of the potato cysteine protease inhibitor group (Cv. Elkana). J Agric Food Chem 53: 5739-5746

RAUSCH T & WACHTER A (2005) Sulfur metabolism: a versatile platform for launching defence operations. Trends Plant Sci 10: 503-509

RAY WK, ZENG G, POTTERS MB, MANSURI AM, LARSON TJ (2000) Characterization of a 12-kilodalton rhodanese encoded by *glpE* of *Ecsherichia coli* and its interaction with thioredoxin. J Bacteriol 182: 2277-2284

RIEMENSCHNEIDER A, NIKIFOROVA V, HOEFGEN R, DE KOK LJ, PAPENBROCK J (2005) Impact of elevated H2S on metabolite levels, activity of enzymes and expression of genes involved in cysteine metabolism. Plant Physiol Biochem 43: 473-483

SAMBROOK J, FRITSCH EF, MANIATIS T (1989) Molecular Cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

SCHENK PM, KAZAN K, RUSU AG, MANNERS JM, MACLEAN DJ (2005) The *SEN1* gene of *Arabidopsis* is regulated by signals that link plant defence responses and senescence. Plant Physiol Biochem 43: 997-1005

SCHLESINGER B, BRETON F, MOCK HP (2003) A hydroponic culture system for growing *Arabidopsis thaliana* plantlets under sterile conditions. Plant Mol Biol Rep 21: 449-456

SHIMADA Y, WU GJ, WATANABE A (1998) A protein encoded by *din1*, dark-inducible and senescence-associated gene of radish, can be imported by isolated chloroplasts and has sequence similarity to sulfide dehydrogenase and other small stress proteins. Plant Cell Physiol 39: 139-143

SPALLAROSSA A, DONAHUE JL, LARSON TJ, BOLOGNESI M, BORDO D (2001) *Escherichia coli* GlpE is a prototype sulfurtransferase for the single-domain rhodanese homology superfamily. Structure 9: 1117-1125

SWIDZINSKI JA, SWEETLOVE LJ, LEAVER CJ (2002) A custom microarray analysis of gene expression during programmed cell death in *Arabidopsis thaliana*. Plant J 30: 431-446

TOOHEY JI (1989) Sulphane sulphur in biological systems: a possible regulatory role. Biochem J 264: 625-632

VENNESLAND B, CASTRIC PA, CONN EE, SOLOMONSON LP, VOLINI M, WESTLEY J (1982) Cyanide metabolism. Fed Proc 41: 2639-2648

WALZ C, GIAVALISCO P, SCHAD M, JUENGER M, KLOSE J, KEHR J (2004) Proteomics of curcurbit phloem exudate reveals a network of defence proteins. Phytochemistry 65: 1795-1804

WEAVER LM, GAN S, QUIRINO B, AMASINO RM (1998) A comparison of the expression patterns of several senescence-associated genes in response to stress and hormone treatment. Plant Mol Biol 37: 455-469

WESTLEY J (1973) Rhodanese. Adv Enzymol 39: 327-368

WILLIAMS JS & COOPER RM (2003) Elemental sulphur is produced by diverse plant families as a component of defence against fungal and bacterial pathogens. Physiol Mol Plant Pathol 63: 3-16

WILLIAMS JS & COOPER RM (2004) The oldest fungicide and newest phytoalexin – a reappraisal of the fungitoxicity of elemental sulphur. Plant Pathology 53: 263–279

WILLIAMS JS, HALL SA, HAWKESFORD MJ, BEALE MH, COOPER RM (2002) Elemental sulfur and thiol accumulation in tomato and defense against a fungal vascular pathogen. Plant Physiol 128: 150-159

WILLIAMS RAM, KELLY SM, MOTTRAM JC, COOMBS GH (2003) 3-Mercaptopyruvate sulfurtransferase of *Leishmania* contains an unusual C-terminal extension and is involved in thioredoxin and antioxidant metabolism. J Biol Chem 278: 1480-1486

WOLFE MD, AHMED F, LACOURCIERE GM, LAUHON CT, STADTMAN TC, LARSON TJ (2004) Functional diversity of the rhodanese homology domain: The *Escherichia coli ybbB* gene encodes a selenophosphate-dependent tRNA 2-selenouridine synthase. J Biol Chem 279: 1801-1809

YANG SH, BERBERICH T, MIYAZAKI A, SANO H, KUSANO T (2003) *Ntdin*, a tobacco senescence-associated gene, is involved in molybdenum cofactor biosynthesis. Plant Cell Physiol 44: 1037-1044

YU C, HOU XL, WU P (2005) The effects of phosphorus, glucose and cytokinin on SEN1 gene expression in Arabidopsis. Zhi Wu Sheng Li Yu Fen Zi Sheng Wu Xue Xue Bao 31: 85-89 (Abstract)

ZHAO Y, DAI X, BLACKWELL HE, SCHREIBER SL, CHORY J (2003) SIR1, an upstream component in auxin signaling identified by chemical genetics. Science 301: 1107-1110