Investigations of the intracellular

localization and the function of

sulfurtransferases in higher plants

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SUMMARY

Sulfurtransferases/rhodaneses (Strs) comprise a group of enzymes widely distributed in archaea, eubacteria, and eukaryota, which catalyze the transfer of a sulfur atom from suitable sulfur donors to nucleophilic sulfur acceptors. In all the organisms analyzed to date small gene families encoding Str proteins have been identified. In this work Strs encoded in the nuclear genome of *Arabidopsis thaliana* were analyzed. 18 proteins were identified in *Arabidopsis* which contain at least one Str signature and were classified into six groups according to their sequence homology. Group I consists of two two-domain Str proteins (AtStr1 and AtStr2), while the proteins in group VI (AtStr14-18) contain only the C-terminal Str signature. Both two-domain proteins from group I showed higher affinity to 3-mercaptopyruvate as substrate than to thiosulfate. To identify thiosulfate Strs or Strs with a different substrate specificity among these proteins and to prove the occurrence of single-domain Strs in *Arabidopsis*, four related proteins of group VI were analyzed. Two proteins from group VI (AtStr16 and AtStr18) were identified as the first single-domain thiosulfate Strs from higher plants.

To continue the determination of the physiological role of both AtStr1 domains and the interdomain linker sequence, several structural analyses were conducted in this work. Overall the physiological role of both domains and the linker remains to be clarified.

To determine the subcellular localization of the *Arabidopsis* Strs from group I and VI, several methods were employed. For AtStr1, mitochondrial localization was obtained by transient expression of fusion constructs with the green fluorescent protein (GFP) in *Arabidopsis* protoplasts whereas AtStr2 was exclusively localized to the cytoplasm. Three members of the single-domain Strs from group VI (AtStr14, 15, and 16) were localized in the chloroplasts, whereas AtStr18 was shown to be cytoplasmic. The remarkable subcellular distribution of AtStr15 was additionally analyzed by transmission electron immunomicroscopy using a monospecific antibody against GFP, indicating an attachment to the thylakoid membrane.

Strs were suggested to be involved in several physiological processes, such as cyanide detoxification or a function during the senescence progress. Therefore, expression and activity of Strs were determined in *Arabidopsis* plants grown under different conditions, but no correlation with the expression pattern of senescence marker genes could be observed. To investigate the possible role in cyanide detoxification, *Arabidopsis* plants

were sprayed with cyanide, but also here the expression pattern and activity levels of Strs remained unchanged in cyanide-treated and control plants. For AtStr1 the evidence for a role in cyanide detoxification from these experiments is low. In the future the construction of knock-out mutants might be helpful to investigate the functional role of Strs

ZUSAMMENFASSUNG

Sulfurtransferasen/Rhodanesen (Strn) umfassen eine Gruppe von in Archaea, Eubakterien und Eukaryoten gleichermaßen verbreiteten Enzymen, die den Transfer eines Schwefel (S)-Atoms von einem geeigneten Donor-Molekül auf nucleophile Schwefel-Akzeptoren katalysieren. In allen bisher untersuchten Organismen konnten Genfamilien identifiziert werden, welche für Strn kodieren. In dieser Arbeit wurden ausschließlich Strn analysiert, die im Genom von *Arabidopsis thaliana* kodiert sind. Es wurden 18 Proteine mit zumindest einer Sulfurtransferase-Signatur in *Arabidopsis* identifiziert und nach ihrer Sequenzähnlichkeit in sechs Gruppen eingeordnet. Gruppe I besteht aus zwei Zwei-Domänen-Strn (AtStr1 und AtStr2), während die Proteine in Gruppe VI (AtStr14-18) nur die C-terminale Sulfurtransferase-Signatur aufweisen. Beide Zwei-Domänen Strn aus Gruppe I zeigen höhere Affinität zu 3-Mercaptopyruvat als Substrat im Vergleich zu Thiosulfat. Um Thiosulfat Strn oder Strn mit einer anderen Substratspezifität innerhalb dieser Proteinfamilie zu identifizieren und das Vorkommen von Ein-Domänen-Strn in *Arabidopsis* zu untersuchen, wurden vier verwandte Proteine aus Gruppe VI analysiert. Zwei Proteine dieser Gruppe (AtStr16 und AtStr18) wurden hierbei als die ersten Ein-Domänen-Strn mit Substratspezifität für Thiosulfat in Pflanzen identifiziert.

Weiterhin wurden in dieser Arbeit verschiedene Strukturuntersuchungen durchgeführt, um mehr über die physiologische Funktion der beiden Domänen von AtStr1 und der Linkersequenz zu erfahren. Hierbei wurde die Bedeutung der Domänen für die Enzymaktivität analysiert. Insgesamt bleibt die physiologische Funktion beider Str Domänen und der Linkersequenz jedoch auch nach diesen Untersuchungen noch zu klären.

Um die Lokalisation der *Arabidopsis* Strn innerhalb der Zelle zu untersuchen, wurden verschiedene Techniken eingesetzt. AtStr1 wurde durch Protoplastentransformation in *Arabidopsis* mit Hilfe von Fusionskonstrukten mit dem grün-fluoreszierenden Protein (GFP) in Mitochondrien lokalisiert, während AtStr2 im Cytoplasma gefunden wurde. Drei Ein-Domänen-Strn aus Gruppe VI (AtStr14, 15 und 16) wurden in Chloroplasten lokalisiert, demgegenüber verbleibt AtStr18 im Cytoplasma. Die auffallende subzelluläre Lokalisation von AtStr15 wurde zusätzlich mit Hilfe von Transmissions-

Elektronenmikroskopie untersucht. Hierbei wurde ein monospezifischer Antikörper gegen GFP eingesetzt, mit dessen Hilfe eine Anlagerung des Proteins an die Thylakoid-Membran nachgewiesen werden konnte.

Für Strn wurden u.a. als physiologische Funktion eine Rolle bei der Cyanid-Entgiftung oder eine Funktion während des Alterungsprozesses der Pflanze vorgeschlagen. Um diesen Hypothesen nachzugehen, wurden Expressionsmuster und Enzymaktivität von Strn bei Pflanzen ermittelt, welche unter verschiedenen Bedingungen angezogen worden waren. Hierbei konnten aber keine Übereinstimmungen mit Seneszens-assoziierten Marker-Genen ermittelt werden. Um die Funktion von Strn bei der Cyanid-Entgiftung zu untersuchen, wurden *Arabidopsis*-Pflanzen mit Cyanid besprüht. Aber auch hierbei konnten keine Übereinstimmungen bei den Expressionsmustern oder der Enzymaktivität zwischen besprühten und Kontrollpflanzen ermittelt werden. Insgesamt ist aufgrund dieser Ergebnisse die Vorstellung einer Funktion von Strn bei der Cyanid-Entgiftung eher gering. In der Zukunft könnte die Konstruktion von "knock-out" Mutanten helfen die physiologische Rolle von Strn zu identifizieren.

Schlagwörter:

Arabidopsis thaliana; Sulfurtransferasen; Cyanid Entgiftung; Intrazelluläre Lokalisation; GFP

ABBREVIATIONS

3-MP	3-mercaptopyruvate
aa	amino acids
ACC	1-aminocyclopropane-1-carboxylic acid
Acc. no.	accession number
AtStr	*Arabidopsis thaliana* sulfurtransferases
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BSA	bovine serum albumine
CaMV	cauliflower mosaic virus
CAS	β-cyano-L-alanine synthase
CLSM	confocal laser scanning microscope
CN^-	cyanide ion
Cys	cysteine
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EST	expressed sequenced tags
GFP	green fluorescent protein
IEF	isoelectric focusing
KCN	potassium cyanide
MeJA	jasmonate methyl ester
NBT	nitroblue tetrazolium
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
PEG	polyethylene glycol
Rhd	rhodanese
RNA	ribonucleic acid

RubisCO	ribulosebisphosphate-Carboxylase/Oxygenase
SAG(s)	senescence associated gene(s)
ST *	sulfurtransferases/rhodaneses
Strs *	sulfurtransferases/rhodaneses
wPS	with peptide signal
woPS	without peptide signal

* The abbreviation changed during the last years.

CONTENTS

CHAPTER 1:

GENERAL INTRODUCTION

What kind of enzymes are sulfurtransferases?

Sulfurtransferases/rhodaneses (Strs) are a group of enzymes widely distributed in all three phyla that catalyze the transfer of sulfur from a donor to a thiophilic acceptor substrate (Westley, 1973). The most studied and best characterized Str is bovine rhodanese (EC 2.8.1.1) which catalyzes, *in vitro*, the transfer of a sulfane sulfur atom from thiosulfate to cyanide, leading to the formation of sulfite and thiocyanate. The overall accepted mechanism can be described corresponding to the following scheme:

$$S_2O_3^{2-} + Rhod \rightarrow SO_3^{2-} + Rhod\text{-}S$$
$$Rhod\text{-}S + CN^- \rightarrow Rhod + SCN^-$$
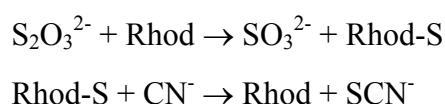
Fig. 1. Reaction mechanism representing the sulfur transfer catalyzed by Strs.

In this reaction the enzyme cycles between two different forms during catalysis, the free enzyme (Rhod), and an enzyme-sulfur intermediate (Rhod-S). The intermediate hosts an extra sulfur atom at this catalytic Cys residue, in contrast to the free enzyme. Crystallographic investigations have shown that the Rhod-S intermediate is characterized by a persulfide bond at the sulfhydryl group of the essential cysteine residue 247 (Gliubich et al., 1998). However, the natural substrates for sulfur donors and acceptors have not been identified yet. Bovine rhodanese structure consists of two domains of similar size and almost identical conformation, connected by a loop at the surface of the molecule (Ploegman et al., 1978). Other important members of the Str family are 3-mercaptopyruvate:(di)thiol Str (EC 2.8.1.2), thiosulfate:thiol Str (EC 2.8.1.3), and thiosulfate:dithiol Str (EC 2.8.1.5), which are closely related, both in the type of reaction catalyzed, and also in their primary amino acid sequence. 3-mercaptopyruvate (3-MP) Strs catalyze a resembling reaction, using 3-MP as sulfur donor instead of thiosulfate. By their entire sequence similarity 3-MP Strs and rhodaneses are confirmed to be evolutionarily related enzymes (Nagahara et al., 1995).

Strs contain structural domains, which are found as tandem repeats, with the C-terminal domain hosting the active site cysteine residue, as single-domain proteins or as members of multi-domain residues (Bordo and Bork, 2002).

In *Arabidopsis thaliana* two cDNA sequences encoding Str proteins were identified, isolated, and characterized (Papenbrock and Schmidt, 2000a, b; Hatzfeld and Saito, 2000; Burow et al., 2002). Both proteins, AtStr1 and AtStr2 show very high homology on the amino acid level to each other and resemble in the decisive amino acids of the 3-MP Strs from mammals. They are based on two nearly identical domains, which are connected with an intermediate linker sequence. In *Arabidopsis* they are encoded on chromosome 1. Their enzymatic characteristics are very similar. Both two-domain Strs show much higher specific activities with 3-MP as sulfur donor than with thiosulfate, but the determined K_m values for both substrates led to the result that the identification of their physiological substrate remains unclear. Thiosulfate Strs like bovine rhodanese or mammalian thiosulfate Str have not been identified in *Arabidopsis* yet. Further on the identification of single-domain Strs, which show high sequence similarity to the C-terminal domain of certain rhodaneses and 3-MP Strs, succeeded only in bacteria. These findings indicate that there is evidence to find single-domain Strs in *Arabidopsis*. A search by database comparisons revealed the existence of 18 *Arabidopsis* proteins containing rhodanese domains, which were classified into six groups according to their sequence homology. The newly identified cDNA sequences from group VI were projected to be isolated and characterized in order to find single-domain (thiosulfate) Strs in *Arabidopsis* in this work. Also the characterization of AtStr1 and AtStr2 (group I) was continued in more detail.

Putative functions of Strs in the organism

In spite of its ubiquity, the determination of the role of sulfurtransferases in biological processes is still largely debated. Many different specific roles like an involvement in cysteine metabolism (Koj et al., 1975), degradation of cysteine (Hannestad et al., 1981), formation in prostethic iron-sulfur proteins (Pagani et al., 1984), a role in sulfate assimilation (Donadio et al., 1990), cyanide detoxification in mammals (Aminlari et al., 1989) or management of the cytotoxicity of reactive oxygen species in aerobic tissues (Nandi et al., 2000) are proposed. In plants, the mobilization of sulfur for transport

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processes out of older leaves was suggested (Papenbrock and Schmidt, 2000b). Strs may also play a role in cyanide detoxification during ethylene-biosynthesis. One possible source of cyanide in higher plants is the biosynthesis of the plant hormone ethylene from 1-aminocyclopropane-1-carboxylic acid (ACC) (Adams and Yang, 1981). During the oxidation of ACC to ethylene one molecule of cyanide is produced for each molecule of ethylene in the presence of iron and oxygen (Yang and Hoffmann, 1984). Further on cyanide is released by other metabolic processes. Results show that extracts from senescent leaves of *Arabidopsis* had higher Str specific activities than extracts from non-senescent tissue. Kasai and coworkers (1996) demonstrated that the rate of ethylene production increased to a maximum and then declined during plant senescence. Cyanogenic glycosides are cyanide compounds produced naturally in many plants, when cyanide is conjugated with saccharides. These glycosides produce hydrogen cyanide when hydrolyzed (Towill et al., 1978). The cyanide ion (CN^-) forms stable but reversible complexes with biologically active metal ions containing enzymes. Such complexation inhibits the activity of enzymes containing these active site metals. In this form it can destruct many physiological processes in cells such as carbon fixation or nitrate reduction.

To circumvent cyanide toxicity, cyanide has to be metabolized to less toxic thiocyanate. It was postulated, that rhodanese and 3-MP Str from rat liver catalyze this metabolism of cyanide to the less toxic thiocyanate via transsulfuration (Nagahara et al., 1999). In contrast, the determination of rhodanese activity in insects that feed on cyanogenic plants supported the idea that rhodaneses may be involved in the tolerance against cyanide, but their primary role is not the detoxification of cyanide in these insects (Urbańska et al., 2002). Even in plants the enzyme has been proposed to have a role in cyanide detoxification, but no correlation between rhodanese activity and cyanide accumulation in plants could be determined (Chew, 1973). The results were confirmed in the way that no correlation between cyanogenesis and rhodanese activity was found (Kakes et al., 1992). Overall the role of Strs in the detoxification of cyanide is contradictory. To estimate the involvement of *Arabidopsis* Strs in cyanide detoxification in more detail, more cyanide treatment experiments will have to be developed. Different expression patterns of several *Arabidopsis* senescence-associated genes (SAGs) during age, dehydration, darkness, abcisic acid, cytokinin, and ethylene treatments have already been successfully analyzed (Weaver et al., 1998). Here several treatment

conditions were created leading to both controlled senescence processes and enhanced cyanide concentrations in the plant.

Detailed determination of the structure

The C-terminal and the N-terminal domain of AtStr1 are connected by a polypeptide chain consisting of 37 extremely hydrophobic amino acids, leaving only little flexibility in this protein area. In comparison to bovine rhodanese (16 amino acids) this interdomain linker sequence is prolonged. Generally in plant sequences the interdomain linker between both domains is quite long in comparison to sequences from prokaryotes and other eukaryotes. Little is known about the linker sequence in Strs. Just the position in the three-dimensional structure near the active site of the enzyme, which is located in the C-terminal domain, was determined for rhodanese with crystallographic analysis (Ploegman et al., 1978). In *Azotobacter vinelandii* the C-terminal RhdA domain hosts the catalytic Cys residue, which is the first residue of a six-amino-acid active-site loop that folds in a cradle-like structure defining the enzyme's catalytic pocket (Bordo and Bork, 2002). Similar results were found in *Arabidopsis,* where the C-terminal domain of AtStr1 showed specific enzyme activity and a removal of the interdomain linker sequence led to a reduction of enzyme activity to about 30% (Burow et al., 2002). Also in *E. coli* RNA polymerase the linker peptide between the globular domains plays an important role in enzyme activity, because insertion, deletion and replacement mutagenesis of several amino acid residues from the linker, led to a dramatic decrease in activity (Fujito et al., 2000). These results demonstrated the importance of the prolonged linker sequence in plant Strs. In contrast the N-terminal domain was described as inactive. Luo et al. (1995) demonstrated, that a N-terminal sequence (residues 2-23) on the surface of the N-terminal domain and the interdomain linker of rhodanese consist of highly resolved amino acids, which are responsible for the stability and the folding of the enzyme. From these results they suggested that the two-domain enzyme structure raises the stability of the whole enzyme and the prolonged linker sequence extended the binding site of the active domain for an unknown substrate. Opposite to this Williams and coworkers (2003) found that the prokaryotic *Leishmania major* 3-MP Str has an unusual C-terminal extension, which might play a role in folding the whole protein. To understand the function of Strs in higher plants, it might be helpful to elucidate the role of the prolonged linker sequence and each single domain.

Localization of Strs in the cell

The intracellular localization may also play a key role for the determination of the metabolic role of Strs *in vivo*. In every organism analyzed so far, the gene products of Strs were localized to different subcellular compartments. In rat e.g. a partially overlapping in the subcellular localization of rhodanese and 3-MP Str were observed. Rhodanese was localized as a membrane-bound protein in mitochondria (Ogata and Volini, 1990), while 3-mercaptopyruvate Str protein was localized to the cytosol and the mitochondria. If Strs play a role in cyanide detoxification, it could be suggested from these results that 3-MP Str detoxifies cyanide more effectively than rhodanese, because rhodanese is localized only in mitochondria. Further on in animals mitochondrial targeting sequences for thiosulfate sulfurtransferases were found at the N-termini (Boggaram et al., 1985). In *E. coli* several Strs were observed, like a two-domain Str localized in the periplasm or a single-domain Str, GlpE, in the cytoplasm (Ray et al., 2000). In *Arabidopsis* the cellular and intracellular localization of AtStr1 and AtStr2 was investigated before (Hatzfeld and Saito, 2000; Papenbrock and Schmidt, 2000a). Tissue-specific distribution of both AtStr1 and AtStr2 was analyzed. Both gene products accumulated in all the tissues examined from flowering *Arabidopsis* plants (flowers, siliques, stems, cauline leaves, rosette leaves and roots) but the highest accumulation was observed in the mature leaves and in roots. To determine the subcellular localization of AtStr1 different methods were employed. Immunoblot analysis of purified organelles from *Arabidopsis* demonstrated that the processed AtStr1 is located in mitochondria and the unprocessed AtStr1 in the cytosol, whereas neither of the proteins could be detected in chloroplasts (Papenbrock and Schmidt, 2000a). However, when sequences of both proteins were labeled with the green fluorescent protein (GFP) from *Aequoria victoria*, which is an *in vivo* marker for protein localization studies, these constructs targeted not only to mitochondria, but also to chloroplasts (Nakamura et al., 2000). Further on in isolated chloroplasts from *Arabidopsis* specific Str activity could be observed. Therefore, the exact localization of these enzymes is still a matter of debate, but there is evidence to find Strs also in chloroplasts or other cell compartments. Another argument for the presence of Strs in chloroplasts was observed in localization studies for a single-domain Str protein (Ntdin) from tobacco, a homologue to a single domain Str from *Arabidopsis* (group VI) with 56.8% identity. Transient expression of GFP fusions with this protein were shown to be

localized in the chloroplasts (Yang et al., 2003). To analyze the function of Strs in *Arabidopsis* and to obtain more information about their putative substrate localization in the metabolic network, the correct subcellular localization of *Arabidopsis* Strs from group I and VI might be one more step towards the elucidation of their function.

Aims and experimental program in this thesis

- Isolation of the five cDNA sequences of group VI from *Arabidopsis* which contain Str domains.

- Heterologous expression in *E. coli* of all five *Arabidopsis* Strs from group VI, purification of the recombinant proteins and characterization of their *in vitro* activities.

- Detailed enzyme kinetic studies for each *Arabidopsis* Str from group VI using the recombinant proteins to identify the main substrate and the respective sulfur acceptor, *in vitro* and *in vivo.*

- Analysis of the protein structure of AtStr1 by isolation of both the C- and the N-terminal domain including and excluding the linker sequence and determination of their specific enzyme activities.

- Functional analysis of AtStr1 by expression analysis under various conditions.

- Subcellular localization of all *Arabidopsis* Str proteins from group I and VI by using different methods, e.g. fusion with the green fluorescent protein (GFP).

CHAPTER 2:

IDENTIFICATION AND CHARACTERIZATION OF SINGLE-DOMAIN THIOSULFATE SULFURTRANSFERASES FROM *ARABIDOPSIS*

Michael Bauer and Jutta Papenbrock

ABSTRACT

Sulfurtransferases/rhodaneses (ST) are a group of enzymes widely distributed in all three phyla that catalyze the transfer of sulfur from a donor to a thiophilic acceptor substrate. All ST contain distinct structural domains, and can exist as single-domain proteins, as tandemly repeated modules in which the C-terminal domain bears the active site, or as members of multi-domain proteins. We identified several ST in *Arabidopsis* resembling the C-terminus of the *Arabidopsis* two-domain ST1 and the single-domain GlpE protein from *E. coli*. Two of them (accession number BAB10422 and BAB10409) were expressed in *E. coli* and purified. Both proteins showed thiosulfate-specific ST enzyme activity.

INTRODUCTION

Sulfurtransferases/rhodaneses (ST) catalyze the transfer of a sulfur atom from suitable sulfur donors to a nucleophilic acceptor. The most studied and best characterized ST is bovine rhodanese (thiosulfate:cyanide ST, EC 2.8.1.1) which catalyzes, *in vitro*, the transfer of a sulfane sulfur atom from thiosulfate to cyanide, leading to the formation of sulfite and thiocyanate (Westley, 1973). ST contain structural well-defined modules. They are found as tandem repeats, with the C-terminal domain hosting the active site cysteine residue, as single-domain proteins or as members of multi-domain proteins (Bordo and Bork, 2002). Although *in vitro* assays show ST activity associated with rhodanese or rhodanese-like domains, specific biological roles for most members of this homology superfamily have not been established (Spallarossa et al., 2001). Proposed roles include cyanide detoxification (Vennesland et al., 1982), sulfur metabolism (Donadio et al., 1990), and mobilization of sulfur for iron-sulfur cluster biosynthesis or repair (Bonomi et al., 1977).

In the *Escherichia coli* genome eight ORFs coding for proteins containing a rhodanese domain bearing the potentially catalytic cysteine have been identified. One of the genes codes for the 12 kDa protein GlpE; relative to the two-domain rhodanese enzymes of known three-dimensional structure, GlpE displays substantial shortening of loops connecting alpha helices and beta sheets resulting in radical conformational changes surrounding the active site. Sequence searches through completed genomes indicate that

GlpE can be considered to be the prototype structure for the ubiquitous single-domain rhodanese module (Vennesland et al., 1982). In all eukaryotic and prokaryotic species investigated so far 3-mercaptopyruvate- and thiosulfate-specific ST were identified (Westley, 1973; Wood and Fiedler, 1953; Colnaghi et al., 2001). So far two *Arabidopsis* cDNA sequences encoding ST proteins were isolated and characterized (Hatzfeld and Saito, 2000; Nakamura et al., 2000; Papenbrock and Schmidt, 2000a, b). Both protein sequences resemble the 3-mercaptopyruvate ST from mammals with respect to decisive amino acid residues. The search for thiosulfate ST in *Arabidopsis* showing homology to the mammalian thiosulfate ST by cDNA library screening had not been successful so far. In the meantime the complete sequence of the *Arabidopsis* genome became available. A comprehensive database screening revealed the existence of at least 18 *Arabidopsis* proteins containing rhodanese domains. We were interested to identify thiosulfate ST among these proteins and to prove the occurrence of single-domain ST in *Arabidopsis*. We could demonstrate that at least two single-domain ST possess thiosulfate ST activity.

MATERIALS AND METHODS

DNA cloning techniques

The EST clones 149L5T7 and 211O17T7 were ordered from the *Arabidopsis* stock center at the Ohio State University and sequenced on both strands using the BIG-Dye Terminator with an ABI 310 (Applied Biosystems). The following primer pair was used to amplify a 411 bp coding sequence from EST 149L5T7: primer 104 (5'-CGG ATC CTC TCA ATC AAT CTC CTC C-3') extended by a *Bam*HI restriction site and primer 105 (5'-CAA GCT TAT TAG CAG ATG GCT CCT C-3') extended by a *Hin*dIII restriction site. For the amplification of the 423 bp coding sequence from EST 211O17T7 primer 108 (5'-GGA TCC GCG GAC GAG AGC AGA GT-3') extended by a *Bam*HI restriction site and primer 109 (5'-CCT GCA GTT GAA GAA GAA GGA GAC G-3') extended by a *Pst*I restriction site were used. A 549 bp cDNA for AAA80303 was amplified from an *Arabidopsis* cDNA library by primer 110 (5'-CGG ATC CGA AAC CAC TGC TTT TAA C-3') extended by a *Bam*HI restriction site and primer 111 (5'-CCT GCA GCT CTT CTA CCG GCA GCT-3') extended by a *Pst*I restriction site.

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 followed by 28 PCR cycles conducted for 45 s at 94°C, 45 s at 55°C, and 45 s at 72°C. The process was finished with an elongation phase of 420 s at 72°C. The amplified PCR fragments were ligated into the expression vector pQE-30 (Qiagen, Hilden, Germany) and introduced into the *E. coli* strain XL1-blue.

Expression and purification of the ST proteins in E. coli

The recombinant proteins were expressed according to the following protocol: After growth of the respective *E. coli* cultures at 37°C to an OD_{600} of 0.6 in Luria Bertani medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl) (Roth) containing ampicillin (100 µg ml^{-1}) (AppliChem, Darmstadt, Germany) induction was carried out for 3 h with 1 mM (final concentration) of isopropyl-β-D-galactoside (AppliChem). Cell lysis was obtained by adding lysozyme (final concentration 1 mg ml^{-1}) (Roth) and vigorous homogenizing using a glass homogenizer and a pestel. The recombinant proteins were purified under non-denaturing or denaturing conditions by affinity chromatography with the nickel affinity resin according to manufacturer's instructions (Qiagen), dialyzed overnight and directly used for enzyme activity measurements. The purity of the protein preparations was checked by SDS polyacrylamide gel electrophoresis (Laemmli, 1970) and subsequent silver-staining. The ST1 protein from *Arabidopsis* (accession number CAB64716) and the N-terminal domain of ST1 (ST1N-term) were expressed and purified as described (Papenbrock and Schmidt, 2000a; Burow et al., 2002).

Enzyme activity measurements

The enzyme assays with recombinant proteins were set up as described (Papenbrock and Schmidt, 2000a; Burow et al., 2002). In general, 1 µg purified recombinant protein was used in each reaction. The determination of the K_m values was done in 1 ml assays under the following conditions: 0.1 M Tris/HCl pH 9.0, 60 mM KCN, and 5 mM 2-mercaptoethanol (Fluka, Taufkirchen, Germany) using 12 different substrate

concentrations in the range of 50 µM to 50 mM of 3-MP (ICN, Eschwege, Germany) or thiosulfate (Fluka), respectively.

Miscellaneous

Protein contents were determined according to Bradford (Bradford, 1976) using bovine serum albumin (Roth) as a protein standard. The DNA and amino acid sequence analyses and prediction of the molecular masses were performed with the programs MapDraw and Protean in DNASTAR (Madison, WI, USA). For the identification of protein domains several programs in http://www.expasy.ch were used. For the prediction of the protein localization different programs were applied (PSORT, Predator and TargetP, http://www.expasy.ch/tools). The multiple sequence alignment was done by ClustalW (http://www.ebi.ac.uk/clustalw). Statistical analysis was performed using the Student method (SigmaPlot for Windows version 7.0). The K_m values were calculated from the non-linear Michaelis-Menten plot using an enzyme kinetic program (SigmaPlot 7.0).

RESULTS

Identification of single-domain ST with a rhodanese signature in Arabidopsis

All members in the ST protein family in eubacteria, archaea, and eukaryotes are unified by characteristic well-defined sequence domains. In PROSITE two signatures were developed for this family: consensus pattern 1 [FY]-x(3)-H-[LIV]-P-G-A-x(2)-[LIVF] (Prosite access number PS00380) and consensus pattern 2 [AV]-x(2)-[FY]-[DEAP]-G-[GSA]-[WF]-x-E-[FYW] (Prosite access number PS00683). Pattern 1 is located in the N-terminal part of two-domain ST, pattern 2 at the C-terminal extremity of the enzyme. Only the C-terminal signature can be found in all ST proteins. We could identify 18 proteins in *Arabidopsis* containing a rhodanese signature. According to their sequence homology they can be classified into six groups (Table 1). In this work we analysed only members of group VI that comprises four closely related proteins and includes one less similar protein. The proteins in this group contain only the C-terminal pattern 2 and possess therefore similarity to the single-domain ST from bacteria (Fig. 1).

The four closely related proteins with the accession numbers AAA80303, BAB10409, AAD03573, and BAB10422 were characterised in more detail. The results of the global

Table 1. Summarizing table containing the members of the ST/rhodanese multi protein family from *Arabidopsis*. The proteins were grouped according to their amino acid sequence similarities using the ClustalW program. An update of the database mining was done on the 8th of march in 2002. The proteins in group I printed in italic letters were analysed previously (Papenbrock and Schmidt, 2000a, b; Burow et al., 2002). The proteins printed in bold letters in group VI were analysed in this study. No., number; aa, amino acids; Mol. mass, molecular mass; Acc. no., accession number in the EMBL database.

	Gene identification	Description	No. ESTs	No. aa	Mol. mass (kDa)	Acc. no.
Group I						
1	At1g16460	thiosulfate sulfurtransferase (ST2)	8	318	34.7	CAB53639
2	At1g79230	thiosulfate sulfurtransferase (ST1)	14	379	41.9	CAB55306
Group II						
3	At5g23060	similar to unknown protein	24	387	41.3	BAB09823
4	At4g01050	hypothetical protein	20	457	49.9	CAB80914
Group III						
5	At5g03455	putative protein	-	132	14.8	CAB83305
6	At1g09280	At1g09280/T12M4_1	9	581	64.7	AAK82550
7	At2g40760	unknown protein	4	522	57.9	AAD32803
8	At1g17850	Contains a Rhodanese-like PF|00581 domain	1	423	48.3	AAF97265
Group IV						
9	At2g42220	hypothetical protein	3	234	25.5	AAB88647
10	At3g08920	unknown protein	2	214	23.8	AAF07833
11	At4g24750	putative protein	4	260	28.7	CAA22988
Group V						
12	At5g19370	putative peptidyl-prolyl cis-trans isomerase	2	299	33.0	AAK43980
13	At5g55130	molybdopterin synthase sulphurylase	5	464	50.6	AAD18050
Group VI						
14	At4g27700	hypothetical protein	2	237	26.3	CAB38282
15	At4g35770	senescence-associated protein	58	182	20.1	AAA80303
16	At5g66040	senescence-associated protein sen1-like protein; ketoconazole resistance protein-like	12	120	12.7	BAB10409
17	At2g17850	putative senescence-associated rhodanese protein	-	150	17.5	AAD03573
18	At5g66170	senescence-associated protein sen1-like protein	6	136	14.9	BAB10422

alignment using the Needleman-Wunsch algorithm underlines the high similarities (on average 50%) between the four proteins analyzed (Fig. 1). For comparison the C-terminal domain of the ST1 (ST1C-term) protein without the linker sequence was included in the analysis; the average similarity to ST1C-term is about 30%. The active site cysteine, the rhodanese signature and 12 further conserved amino acids are the unifying elements. In the global alignment the GlpE protein in *E. coli* shows 20.7% identity and 29.7% similarity with ST1C-term (144 aa), 16.9% identity and 31.8% similarity with ST1N-term (154 aa) whereas the complete ST1 protein (378 aa) has only 7.4% and 13.8% similarity.

```
AAD03573    -MSE--------PKVITIDVNQAQKLLLDSGYTFLDVRTVEEFK-----------KGHVDS  40
BAB10422    -MSQSISSSTKAEEVVSVDVSQAKTLLQSGHQYLDVRTQDEFR-----------RGHCEA  48
AAA80303    -AAEA------VKIPTSVPVRVARELAQAGYRYLDVRTPDEFS-----------IGHPTR  42
BAB10409    -MAEE------SRVPSSVSVTVAHDLLLAGHRYLDVRTPEEFS-----------QGHACG  42
ST1C-term   PISFQT---KFQPHLVWTLDQVKNNMEDPTYQHIDARSKARFDGTAPEPRKGIRSGHIPG  57
                :        .           . :   . :  . :*.*:   .*  .::... ..      **

AAD03573    ENVFNVPYWLYTPQGQEINPNFLKHVSS-LCNQTDHLILGCKSGVRSLHATKFLVSSGFK   99
BAB10422    AKIVNIPYMLNTPQGRVKNQEFLEQVSS-LLNPADDILVGCQSGARSLKATTELVAAGYK  107
AAA80303    --AINVPYMYRVGSGMVKNPSFLRQVSS-HFRKHDEIIIGCESGQMSFMASTDLLTAGFT   99
BAB10409    --AINVPYMNRGASGMSKNPDFLEQVSS-HFGQSDNIIVGCQSGGRSIKATTDLLHAGFT   99
ST1C-term   SKCIPFPQMFDSCNTLLPAEELKKRFDQEDISLDKPIMASCGTGVTACILAMGLHRLGKT  117
             . ..*      .         .: .:..... . :: .* :*  :   :   *   * .

AAD03573    TVRNMDGGYIAWVNK-RFPVKVEHKELKEMKKWADRPMKLQHRQCMYSRRYI  150
BAB10422    KVRNVGGGYLAWVDH-SFPINTEEEEPSAN--------------------  136
AAA80303    AITDIAGGYVAWTEN-ELPVEE-----------------------------  120
BAB10409    GVKDIVGGYSAWAKN-GLPTKA-----------------------------  120
ST1C-term   DVPIYDGSWTEWATQPDLPIESVESSS-----------------------  144
             :    *.:  *. :. :* :   ..
```

Fig. 1. Alignment of single-domain ST sequences from *Arabidopsis*. The sequence of the mature protein AAA80303, the C-terminal part of the ST1-protein (accession number CAB64716, ST1C-term) and the full-length protein sequences of AAD03573, BAB10422, and BAB10409 were aligned using the global alignment algorithm in ClustalW. Identical amino acid residues are marked with an asterisk, similar residues with single dots. The active site cysteine residue and the rhodanese signature are underlined.

According to the number of EST clones isolated so far the four genes differ in their expression levels: For AAA80303 58 EST were identified, for BAB10409 12, for BAB10422 six. For AAD03573 none EST clone is available; therefore the encoding DNA could be a pseudogene. For AAA80303 the existence of an N-terminal extension as transit peptide for plastids was predicted by several programs, whereas the *in silico* analysis for the cell localization of the other proteins was ambiguous.

Specific activities of the single domain ST

Our aim was the expression, purification and determination of ST activity of all four group members. However, for AAD03573 there is no EST clone available and the amplification trials from cDNA libraries have not been successful. The other three proteins were expressed in *E. coli* to high levels, purified by nickel affinity chromatography via the fused 6x His-tag. BAB10422 and BAB10409 could be purified under native conditions, while AAA80303 was purified under denaturing conditions. In the silver-stained SDS gels only single bands were detected in agreement with the predicted sizes (data not shown). Both BAB10422 and BAB10409 were highly active using thiosulfate as sulfane-sulfur donor and cyanide as sulfur acceptor in the classical rhodanese reaction (Fig. 2). The activities with the substrate 3-mercaptopyruvate were very low or almost undetectable. Up to now refolding of AAA80303 back to its active form was not successful.
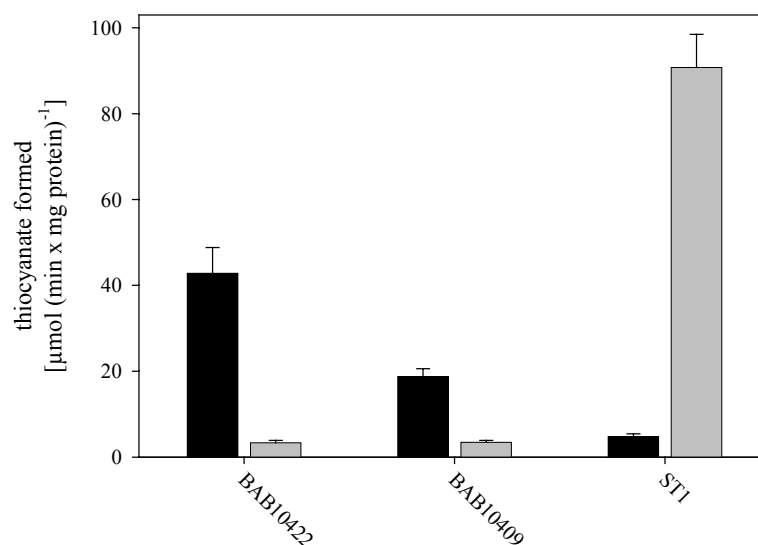
Fig. 2. Enzyme activities of the recombinant proteins. The recombinant STs with the accession numbers BAB10422, BAB10409, and ST1 were purified as described in Materials and Mathods. One µg of recombinant protein was used routinely in each assay. Either thiosulfate (black bars) or 3-mercaptopyruvate (grey bars) was used as sulfur donor. At least two independent isolates were examined and the activity measurements were done in duplicate or triplicate. The mean value of 4 or 6 replicates are shown, the bars represent the respective standard deviation.

The kinetic parameters revealed that both single-domain ST are true thiosulfate ST (Table 2). The K_m values of BAB10422 and BAB10409 for 3-mercaptopyruvate are unphysiologically high because the abundance of 3-mercaptopyruvate in the cell is very low, at least lower than the calculated K_m value as was shown and discussed by our group previously (Papenbrock and Schmidt, 2000b). The velocity of product formation given by the catalytical constant k_{cat} differ for all three ST investigated and characterize ST1 as a 3-mercaptopyruvate ST and BAB10422 as a thiosulfate ST. The catalytic efficiency k_{cat}/K_m includes both the enzyme activity and substrate affinity. The results indicate that although BAB10409 accepted almost exclusively thiosulfate as sulfur donor, the ST1 protein has the potential to function as a better thiosulfate ST than BAB10409. One has to search for a better substrate for BAB10409.

Table 2. Determination of the K_m values for the substrates 3-mercaptopyruvate and thiosulfate, the catalytical constants k_{cat} and the quotients k_{cat}/K_m using the recombinant ST proteins. Enzyme assays were done as described in Materials and Methods; each determination was done four times. The molecular masses (Mol. mass) were calculated for the recombinant proteins excluding the 6x His-tag.

Protein	Mol. mass (kDa)	3-Mercaptopyruvate			Thiosulfate		
		Km (mM)	kcat (s^{-1})	kcat/Km	Km (mM)	kcat (s^{-1})	kcat/Km
ST1	35.5	3.7	54.0	14.6	0.7	2.8	4.3
BAB10422	14.9	35.4	1.2	0.03	1.1	10.7	9.7
BAB10409	12.7	51.7	0.7	0.01	7.4	4.0	0.5

The influence of the ST1N-term on the activity of single-domain ST

As mentioned above both thiosulfate ST contain only the C-terminal rhodanese pattern 2; one could ask the question whether the addition of ST1N-term increases the activity of the single-domain ST. Increasing amounts of the purified ST1N-term (Burow et al., 2002) were added to the ST1C-term and the thiosulfate ST (1 µg ST1C-term or thiosulfate ST plus 1, 2, or 5 µg ST1N-term). As was shown previously the enzyme activity of the ST1C-term could be increased (Burow et al., 2002) whereas the activity of the single-domain thiosulfate ST remained unchanged (Fig. 3). Here the activity of the single-domain ST could not be increased by addition of a protein containing rhodanese pattern 1.
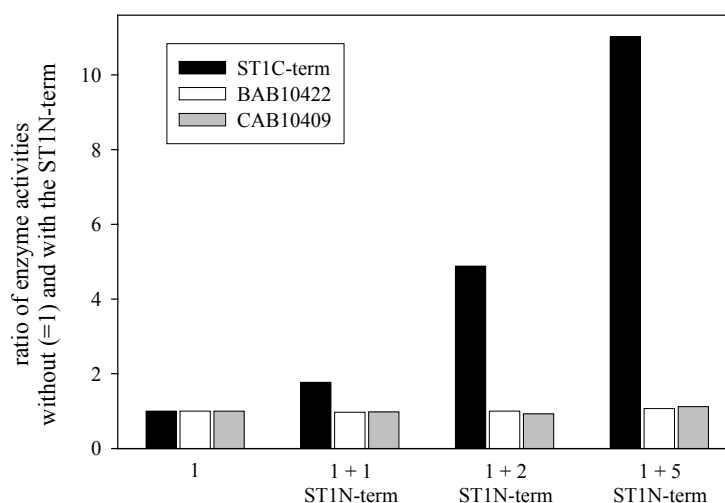
Fig. 3. Combination of the recombinant proteins with the N-terminal domain of the ST1 protein. The enzyme activities of the recombinant proteins (see Fig. 2) were determined without (three columns at the right) and with increasing amounts of the N-terminal domain of the CAB64716 protein (ST1N-term) (1 to 5 times in a µg protein to µg protein ratio). The calculation of the specific activities has been referred to the protein contents of the recombinant full-length proteins. ST1N-term has been expressed separately in *E. coli* and purified as described (Burow et al., 2002). The specific activity of ST1 was determined using 3-mercaptopyruvate as sulfur donor (black bars) whereas the activities of proteins BAB10422 and BAB10409 were determined with thiosulfate as sulfur donor (white and grey bars, respectively).

DISCUSSION

The multi protein family of ST/rhodaneses in *Arabidopsis* comprises at least 18 members including two- and single-domain ST. In our previous experiments we characterized a couple of two-domain ST and demonstrated specificity for 3-mercaptopyruvate (Papenbrock and Schmidt, 2000a, b). In this study the biochemical characterization of two single-domain ST from *Arabidopsis* allowed the identification of thiosulfate-specific ST. So far only in bacteria single-domain ST had been identified and characterized (Spallarossa et al., 2001; Colnaghi et al., 2001; Ray et al., 2000). A prediction of substrate specificity based on the amino acid stretch around the active site cysteine (CRKGVTA for thiosulfate ST and CGS(T)GVTA for 3-mercaptopyruvate ST) as suggested by Colnaghi et al. (2001) could not be applied for the proteins analyzed here. However, the four ST aligned are unified by another highly conserved stretch of amino acids around the active site cysteine residue: [LI]-[IL]-[LIV]-G-C-[KQE]-S-x-[RM]-S. All other *Arabidopsis* ST containing a rhodanese pattern differ in their amino acid sequence in this region (Papenbrock, unpublished). In contrast to the two-domain ST isolated from eukaryotes previously the thiosulfate-specific ST do not contain a second cysteine which was suggested to be involved in acceptor binding close to the active site cysteine (Burow et al., 2002). Obviously the reaction mechanisms differ.

It was shown previously that the enzymatic activity of the *Arabidopsis* ST1 resides in the C-terminal domain but is boosted by the N-terminal domain and the linker peptide in the full length enzyme (Burow et al., 2002). Therefore it was tempting to analyze the effect of additional ST1N-term molecules on the enzyme activity of the single-domain proteins. So far the role of the N-terminal domain and the kind of interactions between both domains are not known; however, the activities of the single-domain proteins could not be enhanced. It was assumed that the two-domain ST evolved from a gene duplication event before the appearance of the *Arabidopsis* genus (Hatzfeld and Saito, 2000). Obviously, both types could evolve independently in the *Arabidopsis* organism.

When comparing the kinetic parameters one has to keep in mind the putative different localizations of the proteins in the cell and different concentrations of the respective substrates. The ST1 protein was shown to be localized in mitochondria (Papenbrock and Schmidt, 2000a) whereas the predictions for the *Arabidopsis* single-domain ST are

plastids, cytosol or nucleus. Currently, we are testing the *in silico* predictions in fusion experiments with the green fluorescent protein.

The more ST with different substrate specificities are identified in diverse organisms the stronger are the demands to clarify their function in the organism. The AAA80303 protein was identified previously as senescence-associated protein (Sen1) (Oh et al., 1996). A homologous protein was isolated from radish (Din1) and was suggested to be the first gene for a chloroplast protein which is negatively controlled by light (Shimada et al., 1998). All members show high similarities to a variety of small stress-related proteins such as sulfide dehydrogenase from *Wolinella succinogenes* (Klimmek et al., 1998), the 11 kDa PspE protein encoded in the phage-shock protein operon (Adams et al., 2002), and the 12 kDa GlpE protein, a member of the sn-glycerol 3-phosphate regulon of *Escherichia coli* (Ray et al., 2000). Recently, for the latter proteins rhodanese activity was demonstrated.

It could be shown that the promotor of the gene *Sen1* encoding AAA80303 contains sequence motifs resembling conserved motifs among stress-inducible genes. Examination of the expression patterns of the *Sen1* gene under various senescing conditions revealed that the *Sen1* and *ST1* gene expressions are associated with *Arabidopsis* leaf senescence (Papenbrock and Schmidt, 2000b; Oh et al., 1996) and *Sen1* is also strongly induced in leaves by abscisic acid or ethephon (Oh et al., 1996). These lines of evidence have to be followed systematically using gene specific probes.

Recent investigations have shown that the rhodanese signatures are structurally related to the catalytic subunit of Cdc25 phosphatase enzymes (Hofmann et al., 1998) and that the two enzyme families are likely to share a common evolutionary origin. An increasing number of reports indicate that rhodanese modules are versatile sulfur carriers that have adapted their function to fulfill the need for reactive sulfane-sulfur in distinct metabolic and regulatory pathways (Bordo and Bork, 2002). The high number of expressed ST in *Arabidopsis* indicates their important role in the organism.

CHAPTER 3:

ARABIDOPSIS SULFURTRANSFERASES: INVESTIGATION OF THEIR FUNCTION DURING SENESCENCE AND IN CYANIDE DETOXIFICATION

Tanja Meyer, Meike Burow, Michael Bauer, Jutta Papenbrock

ABSTRACT

Sulfurtransferases (ST) are a group of enzymes widely distributed in all three phyla that catalyze the transfer of sulfur from a donor to a thiophilic acceptor substrate. In mammals, ST play a role in cyanide detoxification (Westley, 1973); therefore, ST may have a similar function in plants. In higher plants β-cyano-L-alanine synthase (CAS) was also suggested to be involved in cyanide detoxification. The expression and activity of two members in the ST protein family have been shown to increase as the plant ages (Papenbrock and Schmidt, 2000b). The accumulation of *ST1* and *CAS* RNA, the ST and CAS protein levels, and the ST and CAS enzyme activities were determined in *Arabidopsis thaliana* plants grown under different conditions. Senescence-associated processes could be successfully induced by natural aging, by jasmonate methyl ester and darkness in whole plants and detached leaves as demonstrated by the expression of the senescence marker genes *SAG12* and *SAG13*. However, the changes in RNA accumulation and protein levels of ST and CAS did not correlate with the expression of these senescence marker genes; the specific ST and CAS activities either decreased (ST) or increased (CAS). In another experiment, *Arabidopsis* plants were sprayed with cyanide to investigate the role of ST and CAS in cyanide detoxification. The expression of ST and CAS on the RNA and protein levels, and also the enzyme activities remained equal in cyanide-treated and control plants. Incubation with 1-aminocyclopropane-1-carboxylic acid, the precursor of ethylene, increased while fumigation with ethylene decreased expression and activity of ST and CAS. In summary, cyanide does not induce the expression or enhance the activity of ST and CAS in *Arabidopsis*. For both proteins the evidence for a role in cyanide detoxification or induced senescence is low.

INTRODUCTION

Sulfurtransferases comprise a group of enzymes widely distributed in plants, animals, and bacteria that catalyze the transfer of a sulfur atom from a donor molecule to a thiophilic acceptor substrate. Members of the ST family, thiosulfate:cyanide ST (rhodanese, EC 2.8.1.1), 3-mercaptopyruvate:(di)thiol ST (EC 2.8.1.2), thiosulfate:thiol ST (EC 2.8.1.3), and thiosulfate:dithiol ST (EC 2.8.1.5) are closely related, both in the type of reaction catalyzed, and also in their primary amino acid sequence. Several physiological roles have been proposed for these enzymes including cyanide detoxification (Westley, 1973; Vennesland et al., 1982), management of the cytotoxicity of reactive oxygen species in aerobic tissues (Nandi et al., 2000), sulfur metabolism (Westley, 1973; 1981), and mobilization of sulfur for Fe-S cluster biosynthesis or repair (Bonomi et al., 1977; Pagani et al., 1984). Since it was also shown that the expression and the enzyme activity of ST increased with increasing age of *Arabidopsis* plants (Papenbrock and Schmidt, 2000b), one could postulate that ST might also play a role in mobilizing sulfur from senescent leaves into growing leaves, fruits and seeds.

Leaf senescence is a genetically controlled and highly regulated process (Quirino et al., 2000). To study gene expression during senescence processes under controlled conditions, many factors have been chosen to mimic naturally occurring senescence: The transfer of leaves or whole plants into darkness, osmotic stress, extreme temperatures, wounding, ozone, pathogens, and plant hormones such as abscisic acid, ethylene and jasmonic acid (Gan and Amasino, 1997; Quirino et al., 2000). However, differences in gene expression between natural and artificially induced senescence were observed (Becker and Apel, 1993). So called senescence-associated genes (SAG) from *Arabidopsis* showed differential expression patterns in response to stress and hormone treatment (Park et al., 1998; Weaver et al., 1998) as well as under naturally induced senescence (Quirino et al., 2000).

ST may also play a role during senescence by acting to detoxify cyanide produced during ethylene synthesis. It is well known that ethylene is synthesized during ripening of fruits and also during senescence processes in leaves (Abeles et al., 1992). During ethylene biosynthesis, one molecule of cyanide is produced for each molecule of ethylene by the oxidation of 1-aminocyclopropane-1-carboxylic acid (ACC) in the presence of iron and oxygen (Yang and Hoffmann, 1984; Kende, 1993). Although

cyanide can be produced by other metabolic processes, e.g. hydrolysis of cyanogenic glycosides and in amino acid oxidase reactions, the widespread occurrence of ethylene synthesis suggests that this pathway is the principle source of endogenous cyanide in many plants (Kende, 1993). Cyanide is highly toxic for the cell, especially as a potent inhibitor of metalloenzymes, and needs to be detoxified immediately. In plants, at least two proteins could act in its detoxification, ST and β-cyano-L-alanine synthase (CAS, EC 4.4.1.9). ST would catalyze the formation of the less toxic thiocyanate; by mammals this compound is mainly excreted in the urine (Nagahara et al., 1999; Ressler and Tatake, 2001). CAS catalyzes the formation of β-cyano-L-alanine from L-cysteine in the presence of cyanide. β-cyano-L-alanine is further metabolized by the gene product of *NIT4* acting as nitrilase or hydratase to asparagine and aspartic acid, respectively, or can be conjugated to γ-glutamyl-β-cyano-L-alanine (Piotrowski et al., 2001) (Fig. 1). Our interest focuses on the elucidation of the ST function in plants. The expression and activity of ST and CAS during developmentally and artificially induced senescence were followed. In addition, several experimental conditions leading to enhanced cyanide concentrations were created to investigate the putative roles of ST and CAS in cyanide detoxification.
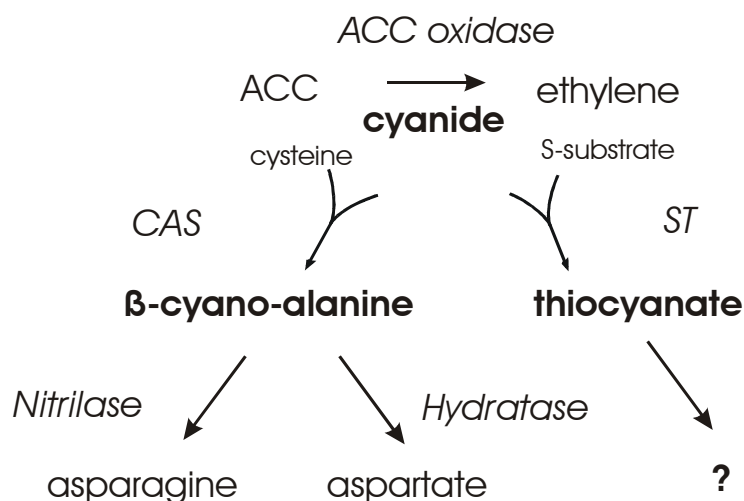
Fig. 1. Scheme of cyanide release during ethylene formation and its putative detoxification pathways in higher plants. *ACC*, 1-aminocyclopropane-1-carboxylic acid; *CAS*, β-cyano-L-alanine synthase; *ST*, sulfurtransferase.

MATERIALS AND METHODS

Growth, treatment and harvest of plants

Seeds of *Arabidopsis thaliana*, ecotype C24, were originally obtained from the Arabidopsis stock center at the Ohio State University. Seeds were germinated on substrate TKS1 (Floragard, 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 m^{-2} s^{-1} (sodium vapor 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. The conditions in the chambers were as following: a 12 h light/12 h dark rhythm at a quantum fluence rate of 50 µmol m^{-2} s^{-1} (TLD 58W/33, Philips) and a constant temperature of 22°C.

To investigate natural senescence *Arabidopsis* plants were grown in the greenhouse for up to 6 weeks, counted from the transfer into the pots, and the parts above ground were cut every week as described before (Papenbrock and Schmidt, 2000b). The oldest leaves were comparable to the S3 stage as defined by Lohmann et al. (1994). A second set of experiments aimed to induce senescence in 4-week-old plants. At this stage the oldest rosette leaves were fully expanded while the inflorescence stem was not yet visible. Seedlings were grown in the way described in the first paragraph, and then transferred for an additional week in climatic chambers. The single *Arabidopsis* plant in each pot was sprayed with 10 ml tap water containing 0.1 mM jasmonate methyl ester (MeJA, Firmenich, Kerpen, Germany) and 0.2 % (v/v) Tween 20. Control plants were treated in parallel with tap water containing 0.2 % (v/v) Tween 20 in a separate but otherwise identical growth chamber. A third set of plants was incubated in the dark in a separate climatic chamber. In parallel, the leaves of 5 plants were cut and incubated with the lower leaf surface upwards in 0.1 mM MeJA in 20 mM phosphate buffer, pH 7.2, or only in 20 mM phosphate buffer in petri dishes. At each time point the detached leaves or the parts above ground from 5 plants were cut, pooled, and frozen in liquid nitrogen. Samples were taken one hour after beginning of the light period shortly before starting the treatment (0 h), and 10, 24, and 48 h after beginning of the treatment.

In a third experiment, single *Arabidopsis* plants were sprayed with 10 ml tap water containing 20 mM cyanide and 0.1 % (v/v) Tween 20. Control plants were grown in a separate growth chamber under the same conditions and treated with the same amount of tap water with 0.1 % (v/v) Tween 20 or tap water alone, respectively. Plants were harvested as described above shortly before starting the treatment (0 h), and 2, 4, 10, 24, and 48 h after spraying.

In a fourth experiment 4-week-old *Arabidopsis* plants were placed into glass containers of 54 l volume which could be closely sealed and were kept in a 12 h light/12 h dark rhythm at a quantum fluence rate of 20 μmol m^{-2} s^{-1} (TLD 58W/33, Philips) and a constant temperature of 20°C. The plants were sprayed with 10 ml of a solution containing 1 or 5 mM 1-aminocyclopropane-1-carboxylic acid (ACC) (Fluka, Germany) and 0.005% (v/v) Tween 20; another set of plants was fumigated with 1 ppm ethylene. Plants were harvested as described, before and at different time points after the beginning of the treatments (1, 6, and 24 h).

RNA extraction and Northern blot analysis

Total RNA was extracted essentially as described (Chomczynski and Sacchi, 1987). RNA samples (15 μg) were separated on 1% denaturing agarose-formaldehyde gels. Equal loading was controlled by staining the gels with ethidium bromide. After RNA-transfer onto nylon membranes, filters were probed with digoxigenin-labeled cDNA probes obtained by PCR. To amplify the ST1 probe sequence specific primers were used (Papenbrock and Schmidt, 2000b). For the PCR labeling reactions of SAG12 and SAG13 the primers KS (5'-TCT AGA ACT AGT GGA TC-3') and SK (5'-CTA GGT GAT CAA GAT CT-3') with the respective cDNA clone as template were used (Weaver et al., 1998), and for amplification of the CAS probe primer 90 (5'-CGG ATC CAA CGC CAA ACG TGA C-3') and primer 91 (5'-CGA CGT CAT CCA CTG AAA CTG G-3') using a cDNA clone provided (Hatzfeld et al., 2000). 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.

SDS/PAGE and Western blot analysis

For the determination of ST and CAS protein steady-state levels in plants, 100 mg plant material was ground with a mortar and pestle in liquid nitrogen to a fine powder. 500 µl sample buffer (56 mM Na_2CO_3, 56 mM DTT, 2% SDS, 12% sucrose, 2 mM EDTA) was added, samples were heated at 95°C for 15 min and cell debris was removed by centrifugation. About 10 µg of total protein was subjected to denaturing SDS gel electrophoresis according to Laemmli (1970) and blotted (Sambrook et al., 1989). A colorimetric detection method using NBT and BCIP was applied. The monospecific antibodies produced against the recombinant Arabidopsis ST1 (Papenbrock and Schmidt, 2000a) and against the purified spinach CAS protein (Hatzfeld et al. 2000) were used for immunodetection.

Enzyme activity measurements

Plant material was ground with a mortar and pestle in liquid nitrogen and the soluble proteins were extracted by adding 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/µl to obtain equal amounts of protein in each assay sample (50 µg). The ST enzyme activity using 3-mercaptopyruvate (3-MP) as sulfur donor was performed as described (Papenbrock and Schmidt, 2000a).

CAS activity was measured by the release of sulfide from L-cysteine in the presence of cyanide (modified from Blumenthal et al., 1968). The assay contained in a total volume of 1 ml: 0.8 mM L-cysteine, 10 mM KCN, and 100 mM Tris/HCl, pH 9.0, and 50 µl protein solution. The reaction was initiated by the addition of L-cysteine. After incubation for 15 min at 30°C the reaction was terminated by adding 100 µl of 30 mM $FeCl_3$ dissolved in 1.2 N HCl and 100 µl 20 mM N,N-dimethyl-p-phenylenediamine dihydrochloride dissolved in 7.2 N HCl (Siegel, 1965). The formation of methylene blue was determined at 670 nm in a spectrophotometer. Solutions with different concentrations of Na_2S were prepared, treated in the same way as the assay samples and were used for the quantification of enzymatically formed H_2S.

Miscellaneous and statistical evaluation

Protein estimation was performed according to Bradford (1976) using bovine serum albumin as a standard. Cyanide was determined by the method described by Guilbault and Kramer (1966). Each incubation experiment was repeated two times. Each type of analysis was done in duplicate with the same frozen plant material. In addition each data point of enzyme activity determinations was obtained by duplicates; for the enzyme activity graphs the 4 replications were combined. Statistical analysis was performed using the Student method (SigmaPlot for Windows version 7.00). Representative Northern and Western blots are shown.

RESULTS

Expression analysis during developmentally induced senescence

As published previously the expression of *Arabidopsis ST1* and *ST2* increased with aging (Papenbrock and Schmidt, 2000b, uppermost box). In the meantime several ST have been identified in *Arabidopsis* (Papenbrock, unpublished); in this paper we would like to concentrate on the expression analysis of *ST1* under several conditions. The *CAS* gene belongs to the *β*-substituted alanine synthase gene family, like the genes coding for *O*-acetyl-L-serine(thiol)lyases (Hatzfeld et al., 2000). The Southern blot analysis using the *CAS* cDNA probe excluded any cross-hybridization with the *O*-acetyl-L-serine(thiol)lyases encoding genes: only single bands were detected when genomic *Arabidopsis* DNA was restricted with different restriction enzymes and hybridized with the *CAS* probe (data not shown). The abundance of *CAS* transcripts was slightly lower in young and very old plants in comparison to the middle-aged plants (Fig. 2). In agreement with the relatively constant *CAS* mRNA expression level in plants of different age neither the CAS protein contents nor the CAS activity increased during naturally occurring senescence as was demonstrated previously (Burandt et al., 2001). To characterize our experimental system in more detail the expression of *SAG12* and *SAG13* was analyzed in the same *Arabidopsis* plants of different physiological ages. The expression of both SAGs increased during aging (Fig. 2). The *SAG12* transcript could only be detected in the 5- and 6-week-old plants, whereas *SAG13* was also present in younger plants at very low levels. Its expression was induced shortly before the

induction of *SAG12* during natural senescence as was demonstrated previously (Lohmann et al., 1994; Weaver et al., 1998). The induction of *SAG12* was defined as the best marker for naturally induced senescence identified so far (Weaver et al., 1998); in our conditions the 5- to 6-week-old *Arabidopsis* plants are just starting their senescence program.
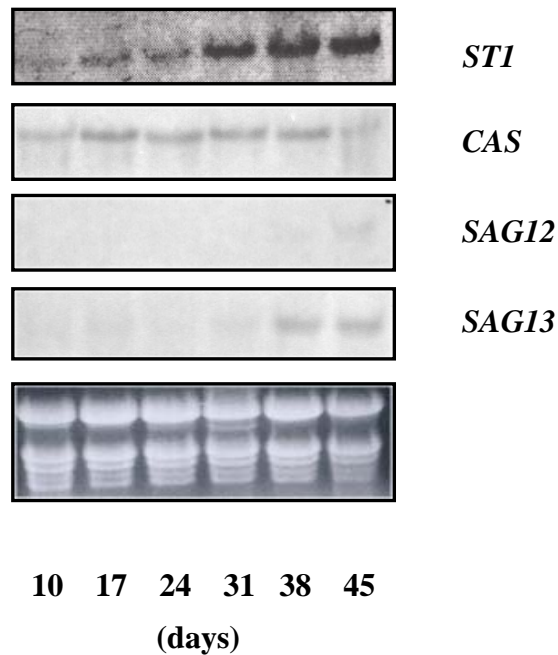
Fig. 2. Northern blot analysis during developmentally induced senescence. *Arabidopsis* plants were grown in the greenhouse for 10 to 45 days, counted from the transfer into the pots, and all plant tissue above ground was used for the extraction of total RNA. 15 µg RNA was loaded in each lane and blotted as indicated in Material and Methods. To prove equal loading of the extracted RNA the ethidium bromide stained gel is shown at the bottom. The cDNAs used for labeling encode ST1, CAS, SAG12, and SAG13, respectively.

Expression analysis and enzyme activities during artificially induced senescence

To find conditions for the specific induction of *ST1* expression, several factors to induce senescence-related processes were tested. First whole plants were sprayed either with MeJA or placed in the darkness. After 10, 24, and 48 h the RNA levels for ST1, CAS, SAG12, and SAG13 were analyzed (Fig. 3A). The mRNA encoding ST1 is almost not detectable in the conditions chosen. *CAS*-mRNA is present at all time points in the
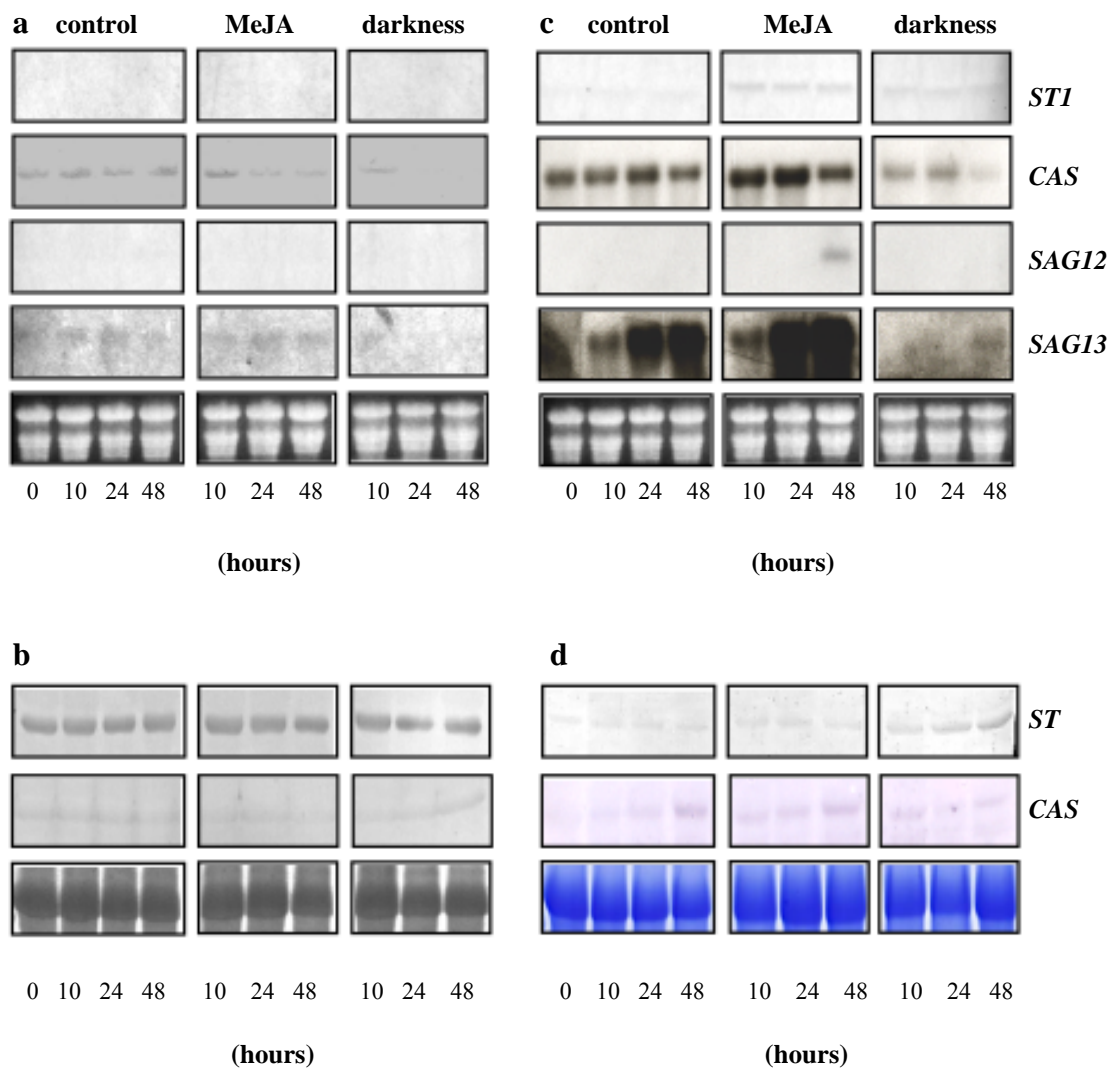
Fig. 3. Northern and Western blot analysis during senescence induced by treatment with MeJA and by darkness. **a** Whole 4-week-old *Arabidopsis* plants grown in single pots were sprayed with 10 ml of a 0.1 mM MeJA and 0.2% (v/v) Tween 20 per plant, control plants were sprayed only with 0.1% (v/v) Tween 20 in H$_2$O. In addition plants of the same age were transferred to darkness. Plant parts above ground were harvested were harvested before starting the spraying (0 h) and after 10, 24, and 48 h and frozen immediately in liquid nitrogen. For each time point 5 plants were combined. Total RNA was extracted from the plant material, 15 µg was loaded in each lane and blotted as indicated in Material and Methods. To prove equal loading of the RNA the ethidium bromide stained gel is shown at the bottom. The cDNA used for labeling encode ST1, CAS, SAG12, and SAG13. **b** The same plant material described in **a** was used for Western blot analysis. Total protein extracts were prepared, separated on SDS-gels and blotted onto nitrocellulose membranes. Monospecific antibodies recognizing the ST and CAS proteins, respectively, were used for the immunoreaction. The Coomassie-stained gel loaded with the same samples is shown in the lowest panel to demonstrate loading of equal protein amounts. **c** The leaves of five 4-week-old *Arabidopsis* plants were cut and incubated in 20 ml solution of 0.1 mM MeJA and 0.2% (v/v) Tween 20 in petri dishes or incubated in 0.1% (v/v) Tween 20 either in the dark or in the light (control). The detached leaves were harvested before starting any incubation (0 h) and after 10, 24, and 48 h and frozen immediately in liquid nitrogen. mRNA contents were analyzed as described in **a**. **d** The same plant material analyzed for mRNA contents in C was used for Western blot analysis as described in **b**.

controls, then the expression in MeJA-treated tissue is repressed 1 and 2 d after beginning of the treatment. The *CAS*-mRNA is present up to 10 h in the dark treatment, afterwards the expression decreases below the detection limit under these experimental conditions. *SAG12*-mRNA levels were undetectable, similar to and as expected from the literature (Weaver et al., 1998), whereas *SAG13* was expressed at low levels during the whole experiment. In the Western blot analysis the ST and CAS protein levels remained constant under the conditions analyzed in comparison to the control plants (Fig. 3B).

In the next experiment leaves were cut from 4-week-old *Arabidopsis* plants and incubated either in phosphate buffer with or without MeJA in the light or in phosphate buffer in darkness for 10, 24, and 48 h. In detached leaves the *ST1*-mRNA was present under all conditions analyzed (Fig. 3C). The transcripts levels were higher after treatment with MeJA and in the dark in comparison to the controls. In detached leaves the expression of *CAS* was induced by the MeJA in comparison to the controls; these results are in contrast to the expression levels obtained with whole plants. The incubation of detached leaves with MeJA led to an increase of *SAG12* expression 2 days after incubation, whereas the expression of the second marker gene increased in the controls and, more drastically by the incubation with MeJA. However, the influence of darkness repressed the gene expression of *SAG13* in detached leaves. The Western blot analysis of the same plant material demonstrated an increase of ST1 protein levels in continuous darkness whereas the CAS protein levels remained almost unchanged (Fig. 3D).

To complete the analysis, the 3-MP dependent specific activity of ST and the specific activity of CAS were analyzed in this same plant material. After the treatment of whole plants the ST activity did not change significantly during the different treatments in comparison to the controls (Fig. 4A, left). The specific CAS activity was reduced after watering with a MeJA solution and prolonged darkness in comparison to the untreated control plants (Fig. 4A, right). In detached leaves the ST activity decreased significantly after 10, 24 and 48 h in the control as well as in the treated leaves while the specific CAS activity remained almost constant after 10 and 24 h and increased drastically after 48 h (Fig. 4B).

a

b
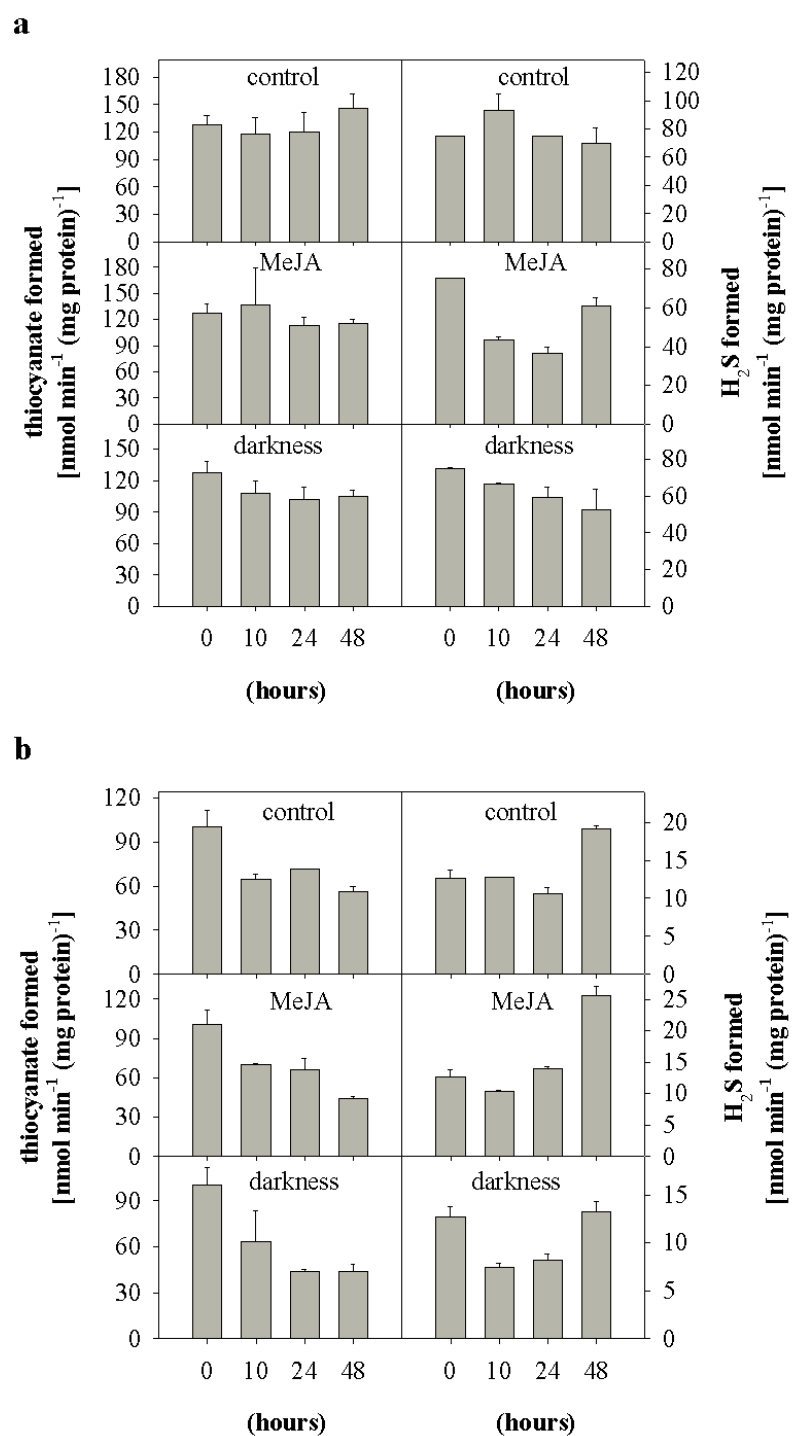
Fig. 4. Determination of enzyme activities during senescence induced by treatment with MeJA and by darkness. **a** The same plant material harvested as described in Fig. 3a (whole plants were sprayed) was used for the determination of ST activity using 3-MP as substrate (graphs on the left) and the CAS activity (graphs on the right). **b** The same plant material harvested as described in Fig. 3c (detached leaves were incubated) was used for the determination of ST activity using 3-MP as substrate (graphs on the left) and the CAS activity (graphs on the right).

Expression analysis and enzyme activities after application of cyanide

Arabidopsis plants were sprayed with a solution containing 20 mM KCN and 0.1% (v/v) Tween 20. In addition to the control of H_2O, a second control was applied (H_2O, 0.1% (v/v) Tween 20) because pre-tests revealed effects by the detergent. By visual examination the plants did not show any damage after cyanide spraying in comparison to both sets of control plants. In comparison to the controls the *ST1*-mRNA expression levels decreased when treated with cyanide (Fig. 5A, upper box) whereas the *CAS*-mRNA levels did not change significantly in controls and cyanide treated plants (Fig. 5A). *SAG12*-mRNA accumulation did not reveal clear results. The *SAG13*-mRNA expression pattern was similar in all treatments. The Western blot analysis did not show any significant differences in the ST1 and CAS protein contents between treated and untreated plants (Fig. 5B). Neither the ST nor the CAS activities were enhanced by the cyanide treatment in comparisons to controls at any of the time points (data not shown).

Because it is well known that cyanide tends to evaporate from solution into the air at a broad physiological pH range the cyanide contents of the tissue were measured to determine if the spraying treatment was actually successful (Fig. 6). The cyanide content in the treated material was about 5 times higher than in untreated plant material. Although the plants were carefully washed before analysis it is not known whether the cyanide measured is located in the plant cells or at the leaf surface. We have to assume from spraying experiments with other compounds in Tween 20-containing solutions that at least some of the highly concentrated cyanide solution sprayed onto the leaves has been internalized. In comparison to the cyanide solution sprayed (20 mM), only about one part out of 1,000 could be recovered (about 20 µM).
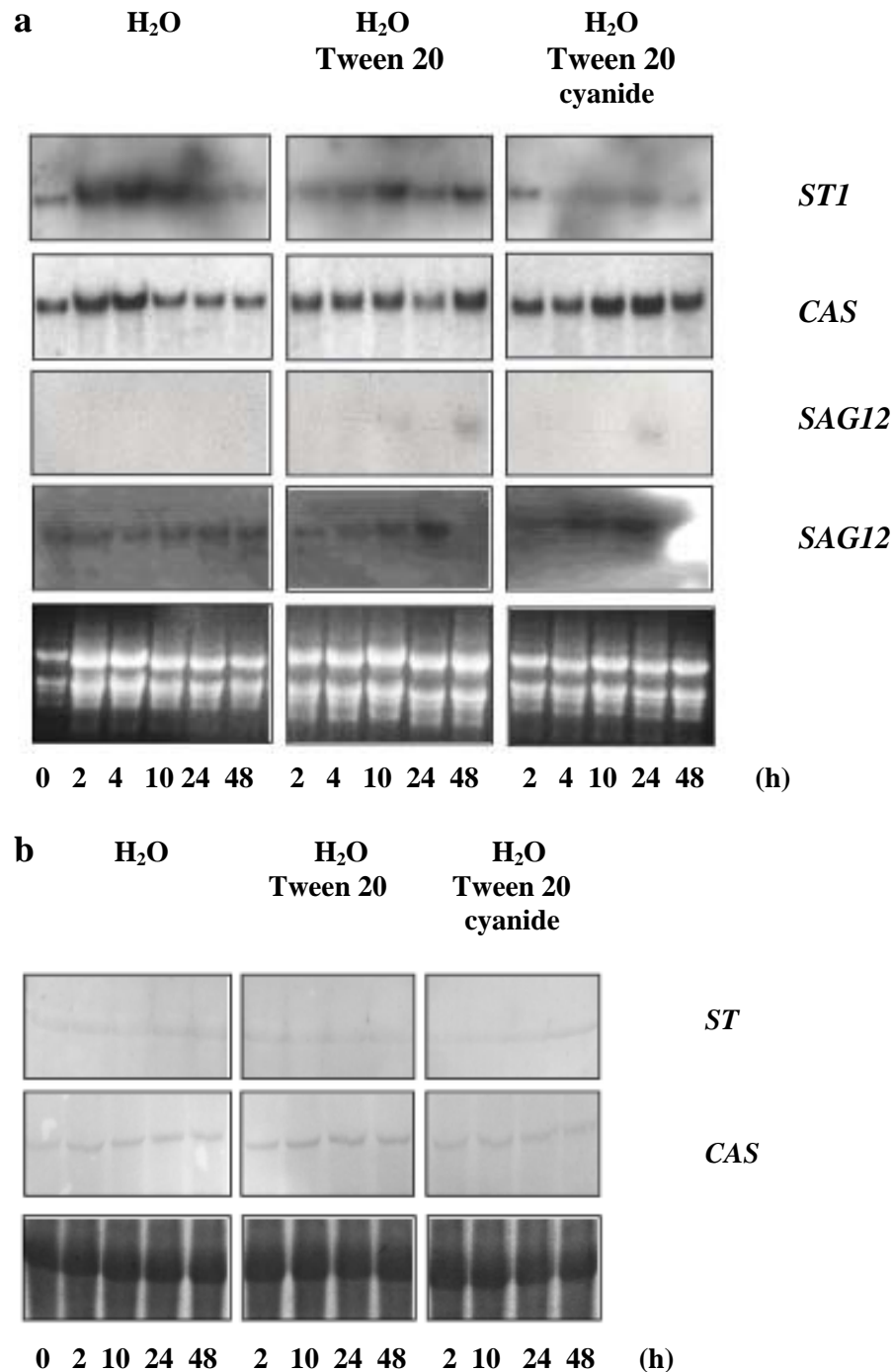
Fig. 5. Northern and Western blot analysis after treatment with cyanide. **a** 4-week-old *Arabidopsis* plants were sprayed with 10 ml of H$_2$O, with 0.1% (v/v) Tween 20 in H$_2$O or with 20 mM cyanide and 0.1% (v/v) Tween 20. The control (0 h) was taken shortly before starting the treatment. Plant parts above ground were cut after 2, 4, 10, 24, and 48 h and frozen in liquid nitrogen. Total RNA was extracted, 15 μg were separated on denaturing gels, blotted and hybridized with cDNA probes encoding ST1, CAS, SAG12, and SAG13. To control equal loading the ethidium bromide stained gels are shown at the bottom. **b** The same plant material as described in **a** was used for Western blot analysis using the monospecific antibodies against ST and CAS. The Coomassie-stained gel loaded with the same samples used for the Western blot is shown in the lowest panel to demonstrate equal protein amounts in each lane.
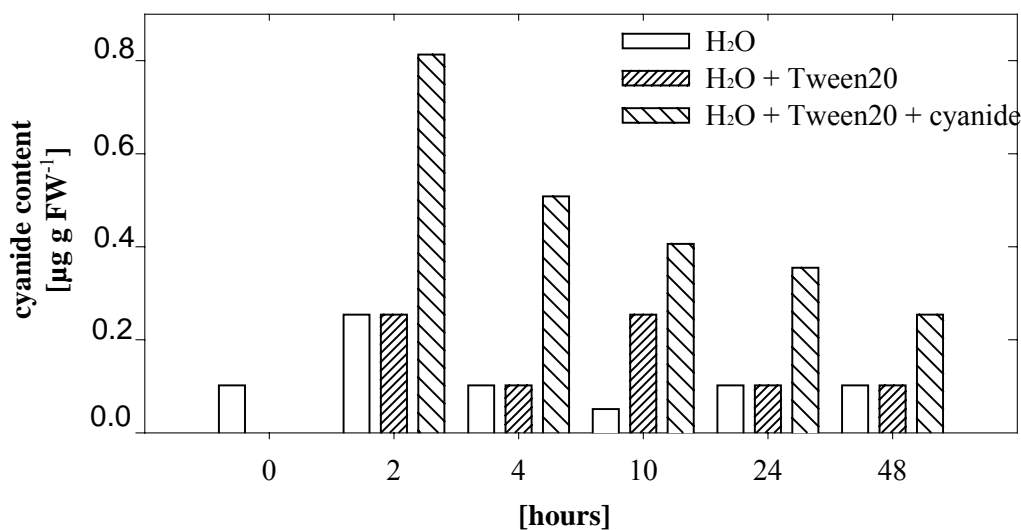
Fig. 6. Determination of the cyanide content in the leaves of the *Arabidopsis* plants treated with cyanide. Plants were treated as described in Fig. 5a. The cyanide contents in this plant material were estimated by a modified method according to Guilbault and Kramer (1966).

Analysis of ST and CAS expression and activity after application of ACC and ethylene

By the application of ACC we expected an increase of both cyanide and ethylene synthesis; whereas by the application of ethylene only the influences of enhanced ethylene concentrations become effective. 4-week-old *Arabidopsis* plants were incubated with 1 or 5 mM ACC, or 1 ppm ethylene as described in Material and methods. Treated plants were cut after the time points indicated and directly frozen. The expression of *ST1* was increased by the application of 1 and even more by 5 mM ACC (Fig. 7A), whereas the application of ethylene alone did not increase the expression of *ST1*. The *CAS* expression levels did not change; *SAG12* expression was induced by 5 mM ACC after 24 h and by ethylene treatment whereas the *SAG13* expression was slightly induced by all treatments in comparison to the control. Again on the protein level no differences could be detected in the Western blot analysis (Fig. 7B).

Finally, the influence of ACC and ethylene on the specific ST and CAS enzyme activities was determined (Table 1; 2). Neither 1 nor 5 mM ACC enhanced the specific activities of either enzyme significantly. However, fumigation with ethylene did significantly decrease the ST activity to less than 50% in comparison to the controls. (Table 1) The CAS activities were reduced to about 70% in comparison to the controls (Table 2).
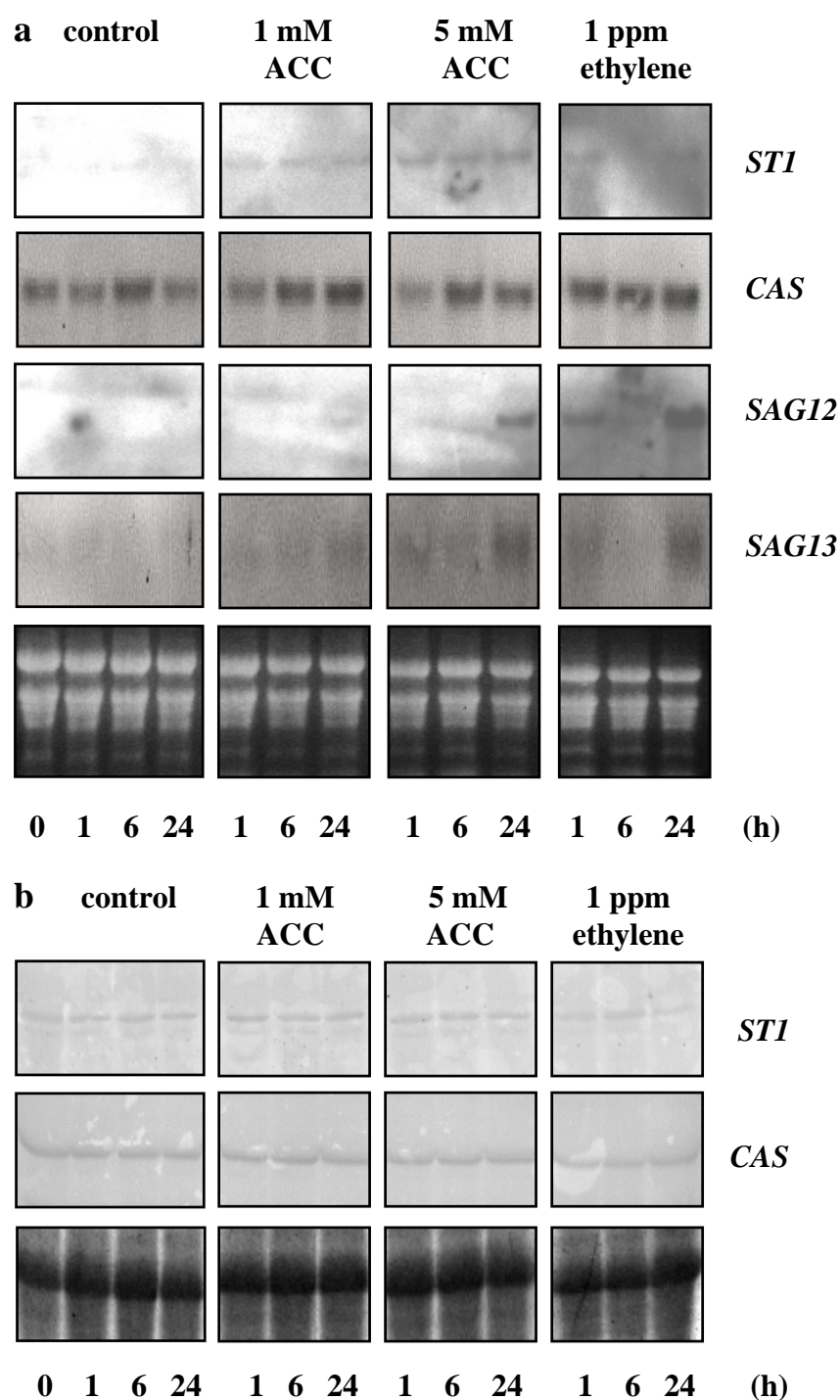
Fig. 7. Northern and Western blot analysis after treatment with 1-aminocyclopropane-1-carboxylic acid (ACC) and ethylene. **a** 4-week-old *Arabidopsis* plants were placed in glass chambers and either sprayed with ACC (1 and 5 mM) or fumigated for 15 min with 1 ppm ethylene. The plant parts above ground of 5 plants were harvested shortly before starting the treatments and 1, 6, and 24 h after the treatment. Northern blot analysis was done as described in Material and Methods. Total RNA was probed with cDNAs coding for ST1, CAS, SAG12, and SAG13. **b** The same plant material was subjected to a Western blot analysis using antibodies directed against ST1 and CAS.

Table 1. ST enzyme activities in *Arabidopsis thaliana* plants treated with ACC or ethylene in comparison to control plants. The ST activity was determined by using 3-mercaptopyruvate as sulfur donor and cyanide as sulfur acceptor, and measuring the formation of thiocyanate. Different letters indicate significant differences between enzyme activities at $\alpha = 0.05$ (Student's test).

	Thiocyanate formed [nmol (min x mg protein)$^{-1}$]			
	H$_2$O	1 mM ACC	5 mM ACC	1 ppm ethylene
0 h	82.5 ± 14.1^a			
1 h	104.1 ± 22.0^a	95.6 ± 0.3^a	80.6 ± 13.8^a	47.7 ± 12.1^b
6 h	86.2 ± 20.2^a	87.9 ± 14.0^a	74.3 ± 8.2^a	41.7 ± 1.1^b
24 h	93.5 ± 8.7^a	76.9 ± 15.1^a	83.0 ± 11.0^a	45.9 ± 7.1^b

Table 2. CAS enzyme activities in *Arabidopsis* plants treated with ACC or ethylene in comparison to control plants. The CAS activity was determined by measuring the release of H$_2$S in the reaction of L-cysteine and cyanide. Different letters indicate significant differences between enzyme activities at $\alpha = 0.05$ (Student's test).

	H$_2$S formed [nmol (min x mg protein)$^{-1}$]			
	H$_2$O	1 mM ACC	5 mM ACC	1 ppm ethylene
0 h	142.2 ± 1.0^a			
1 h	149.6 ± 0.1^a	132.7 ± 4.9^a	141.5 ± 24.5^a	105.9 ± 1.8^b
6 h	141.9 ± 8.7^a	132.4 ± 1.7^a	126.5 ± 8.3^a	103.0 ± 11.8^b
24 h	145.2 ± 1.4^a	126.6 ± 11.7^a	147.6 ± 1.0^a	122.3 ± 0.9^b

DISCUSSION

Our aim is to elucidate the function of ST in higher plants. In the experiments described here the involvement of ST in senescence-associated processes and cyanide detoxification was investigated. Recently, similarities of ST to senescence-associated proteins were found by sequence comparisons (Papenbrock and Schmidt, 2000b). The *in silico* data supported the experimental data because both expression of *ST1* and *ST2*, and MST activity were higher in older than in younger *Arabidopsis* plants (Papenbrock and Schmidt, 2000b). The analysis of the CAS enzyme was included in our investigation because of its postulated role in cyanide detoxification (Manning, 1988; Grossmann, 1996).

It is well known that the initiation of the senescence syndrome is induced by different autonomous or environmental signals which are possibly interconnected to form a regulatory network and demonstrate the plasticity of leaf senescence (Gan and Amasino, 1997; Quirino et al., 2000). In a number of experiments performed previously about 30 SAGs were isolated and grouped into two classes. The class I SAGs were only expressed during senescence processes whereas the expression of class II SAGs increased from a basal level to higher levels during senescence. Most of the SAG gene products belong to several groups of degradative enzymes. For example, *SAG12* encodes a cysteine protease and *SAG13* a short chain alcohol dehydrogenase. The induction pattern of *SAG12* was characterized to be specific for naturally induced senescence whereas it could not be induced by darkness, osmotic stress and hormone treatment in whole plants. *SAG13* expression levels were enhanced by ozone, hormones like ethylene, wounding, and to a certain extent by darkness (Weaver et al., 1998; Miller et al., 1999; Noh and Amasino, 1999).

To investigate the factors influencing expression and activity of ST, *Arabidopsis* plants were confronted with different artificial senescence-inducing conditions. In our experiments the expression levels of *SAG12* and *SAG13* were comparable to the results reported in the literature; in summary, the expression of *ST1* deviated from the expression of both class I and class II senescence markers. Its expression pattern differed from the typical *SAGs* and might be regulated by a different signal cascade.

Interestingly, the treatment of whole plants with MeJA and darkness did not induce expression of *ST1* whereas the incubation of cut leaves raised the *ST1*-mRNA contents.

The cutting and therefore wounding of leaves, and also the continuous incubation in MeJA in contrast to one time watering could cause an increased expression. On the other hand, the *CAS* expression was decreased by MeJA and darkness similar to results obtained with potato tubers (Maruyama et al., 2001). In general, wounding results in an endogenous formation of jasmonic acid (Kramell et al., 1997) supporting the effect of exogenous factors. Assuming a role for ST in transport processes (Papenbrock and Schmidt, 2000b) the expression could be induced by cutting of leaves or branches. Recently, the proteins in the phloem sap of *Cucurbita* seedlings were separated by 2D-gel electrophoresis and their sequences were determined by mass spectrometry. One of the analyzed proteins showed high homology to ST from *Arabidopsis* (Haebel and Kehr, 2001). These results support a role of members of the ST protein family in mobilizing and transporting sulfur. In future experiments it might be interesting to investigate natural senescence under limiting sulfur supply in wild-type and transgenic *ST* antisense plants.

ST has been suggested to be involved in cyanide detoxification for a long time, especially in animals (Westley, 1973; 1981). However, reports did not reveal clear evidence for the involvement of ST, at least in plants. For example, in the investigation of 9 non-cyanogenic and 3 cyanogenic species done by Chew (1973) it was shown that there appeared no correlation between ST activity and the cyanogenic nature of the plant. The cyanide detoxification by CAS activity was also reported for animals but the protein has not been identified so far (Way et al., 1988; Meyers and Ahmad, 1991). The results for the plant kingdom concerning the detoxification of cyanide by CAS are ambiguous: In non-cyanogenic plants, like *Arabidopsis*, cyanide is mainly released during the biosynthesis of ethylene (Goudey et al., 1989) that is formed during senescence and fruit ripening (Manning, 1988; Yip and Yang, 1988). The ACC oxidase that releases cyanide is a cytosolic protein (Reinhardt et al., 1994) whereas the putative cyanide detoxification proteins ST and CAS are localized in mitochondria (Hatzfeld et al., 2000; Papenbrock and Schmidt, 2000a; Warrilow and Hawkesford, 2000). To our knowledge there exist no other cyanide generating reactions in mitochondria. One has to assume that the cyanide removal in cell compartments outside the mitochondria is less efficient, resulting in transiently elevated cyanide in cytosol or chloroplasts. However, the dissociation of cyanide is pH-dependent, therefore cyanide might be able to diffuse from the cytosol into mitochondria. The measurement of organelle-specific concentrations for cyanide would be helpful to follow this speculation. Another

interesting point concerns the kinetic properties of the CAS protein. For CAS a K_m value for cyanide was determined to be 0.5 mM (Yip and Yang, 1988) or 5 mM (Hatzfeld et al., 2000) whereas the cyanide concentration in the plant tissue was calculated to be 0.2 µM and appeared to be kept well below the non-toxic level of approximately 1 µM (Yip and Yang, 1988; Grossmann, 1996). On the other hand one has to keep in mind that the enzymatic activities *in vitro* do not always represent the *in vivo* situation. One could also speculate about further so far unidentified routes for the detoxification of cyanide in the cytosol. So far the role of the vacuole in sequestering cyanide from the cytosol was also not investigated.

The product of CAS, *β*-cyano-L-alanine, is further metabolized in *Arabidopsis* by the NIT4 protein acting as *β*-cyano-L-alanine hydratase/nitrilase to aspartate and asparagine, respectively (Piotrowski et al., 2001; Fig. 1), major amino acids which are subsequently transported to sink tissues. CAS and NIT4 activities are in the same range [CAS about 200 pkat (mg protein)$^{-1}$, Burandt et al., 2001; NIT4 between 25 and 250 pkat (mg protein)$^{-1}$, Piotrowski et al., 2001]. The localization of NIT4 is probably cytosolic (M. Piotrowski, personal communication). Because of the relatively high specific activities one could also assume a biosynthetic role for the pathway CAS/NIT4 by providing the cell with C4 bodies.

In experiments done previously, *Pisum* plants were exposed to ethylene, therefore the cyanide-releasing ACC oxidase step was circumvented, and still a several fold increase of CAS activity was determined supported by *de novo* biosynthesis of CAS. The incubation of potato tubers with ethylene suggested that the hormone increased CAS protein levels by enhancing its translation (Maruyama et al., 2001). Thus ethylene promotes the capacity of several plant tissues to metabolize cyanide. However, its continuous presence is required to sustain elevated levels of CAS activity (Goudey et al., 1989). This fact might explain the results obtained by short-term incubation in our experiments where fumigation by ethylene using *Arabidopsis* plants decreased expression and activities of ST and CAS (Fig. 7A, Table 1). In addition, the influence of ethylene might be organ- or species-specific. For example, the abundance of ST genes is not known for other species except *Arabidopsis*. Interestingly, gaseous cyanide given for 24 h had no measurable effect on the levels of CAS activity (Goudey et al., 1989). These results support a role for ethylene in regulating the capacity of plant tissues to metabolize cyanide. The analysis of mutants in ethylene biosynthesis could be a promising tool to clarify the connection between CAS activity and ethylene.

For ST the evidence for an involvement in cyanide detoxification as the only role in the metabolism is very low. Raising the cyanide content by factor 5 has neither an effect on the expression of *ST1*-mRNA and the ST protein contents nor on the ST enzyme activity in comparison to controls. The treatment with ACC increased *ST1* expression slightly while the enzyme activity remained unchanged. Cyanide levels in *Manihot* are about 4,000 times higher than in *Arabidopsis* which clearly classify *Arabidopsis* into the group of non-cyanogenic plants. Still the ST activity in the cyanogenic *Manihot* is only about 5 times higher than in *Arabidopsis* (data not shown). The only explanation would be that the constitutively expressed levels of *ST* are sufficient for the cyanide detoxification in the plant organism. For a clarification of the results the determination of the molar ratios of ACC, cyanide, ethylene, thiocyanate, and *β*-cyano-L-alanine in *Arabidopsis* wild-type and *ST*-mutants is a challenging task in the future.

CHAPTER 4:

ARABIDOPSIS SULFURTRANSFERASES: INVESTIGATION ON THEIR ROLE IN THE ORGANISM

Tanja Meyer, Meike Burow, Michael Bauer, Jutta Papenbrock

INTRODUCTION

Sulfurtransferases/rhodaneses are a group of enzymes widely distributed in plants, animals, and bacteria that catalyse the transfer of a sulfur atom from a donor molecule to a thiophilic acceptor substrate (Westley, 1973). The exact function of sulfurtransferases (ST) in the plant organism is not known so far. Several functions were suggested: such as a role in cyanide detoxification in analogy to mammalian ST (Westley, 1981), the detoxification of free oxygen radicals in a thioredoxin reductase reaction (Nandi et al., 2000), providing sulfur for biosynthetic reactions, e.g. for Fe-S cluster (Bonomi et al., 1977), mobilisation and transport of reduced sulfur, especially during aging of plants (Papenbrock and Schmidt, 2000b). Here, we would like to investigate the putative role of ST1 in cyanide detoxification in the non-cyanogenic plant *Arabidopsis*.

MATERIALS AND METHODS

Experimental design: 4-week-old *Arabidopsis* plants grown on soil were sprayed with a 20 mM cyanide solution and compared with control plants. At the time points indicated in figure 1 the parts above ground were cut and frozen in liquid nitrogen. The expression on the RNA and protein levels, and the respective enzyme activities were determined as described (Papenbrock and Schmidt, 2000b) for ST and for β-cyano-L-alanine synthase (CAS), another enzyme supposed to be involved in cyanide detoxification in higher plants. The cyanide contents were determined according to Guilbault and Kramer (1966).

RESULTS

In comparison to the expression in control plants the amounts of *ST* and *CAS* RNA levels were unimpaired (Fig. 1). Also on the protein levels as demonstrated in Western blot analysis using monospecific antibodies the levels of ST and CAS did not change after the plants were treated with cyanide (data not shown). The enzyme activities remained almost constant in control and in the cyanide treated plants or even decreased after cyanide treatment (Table 1). However, it was shown that the cyanide contents in the plants were raised at least 5times after being sprayed with cyanide.
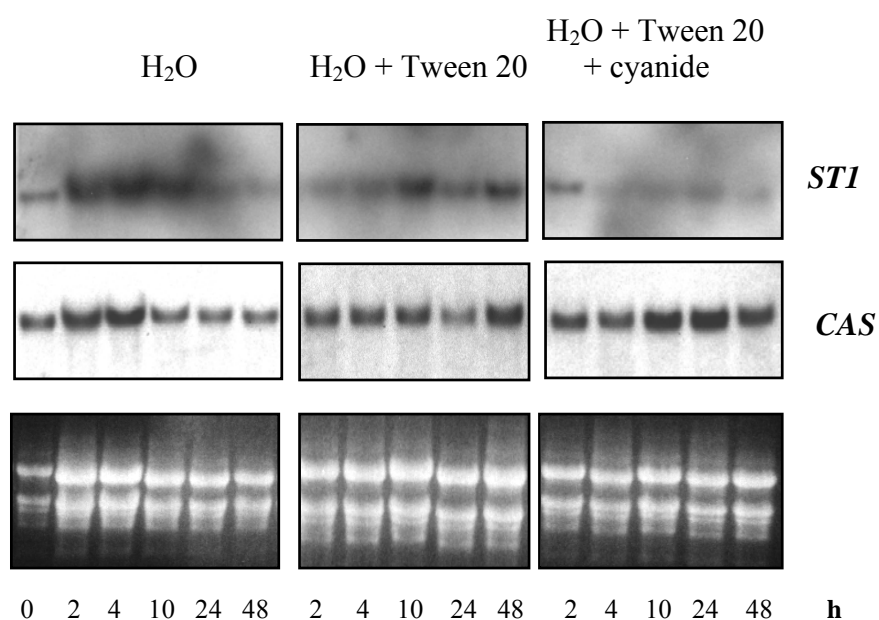
Fig. 1. Northern blot analysis. 4-week-old *Arabidopsis* plants were grown on soil and sprayed with 10 ml of H_2O (left), H_2O and 0.1 % (v/v) Tween 20 (middle), and H_2O, 0.1 % (v/v) Tween 20 and 20 mM cyanide (right). Total RNA was extracted, separated and blotted. The membranes were hybridized with *ST1* and *CAS* DNA probes. The boxes at the bottom show the respective ethidium bromide-stained gels to demonstrate equal loading.

Table 1. Cyanide contents and determination of enzyme activities in plants treated with 20 mM cyanide solution in comparison to untreated plants. The ST activity was determined by using 3-mercaptopyruvate as sulfur donor and cyanide as sulfur acceptor. The CAS activity was determined according to Blumenthal et al. (1968). The values for the second control, H_2O and 0.1 % Tween 20, are very similar to the H_2O control and are therefore not shown. The standard error was always below 5%. FW, fresh weight.

	cyanide		thiocyanate formed		sulfide formed	
	$[\mu g \ (g \ FW)^{-1}]$		$[nmol \ (min \ x \ mg \ protein)^{-1}]$		$[nmol \ (min \ x \ mg \ protein)^{-1}]$	
	H_2O	20 mM KCN^-	H_2O	20 mM KCN^-	H_2O	20 mM KCN^-
0 h	0,11	-	48,6	-	67,6	-
2 h	0,05	0,81	60,5	37,8	50,5	40,7
4 h	0,13	0,51	52,3	44,5	42,9	38,2
10 h	0,05	0,41	50,9	41,7	49,1	36,9
24 h	0,16	0,36	59,9	50,3	57,1	47,9
48 h	0,14	0,25	54,0	44,5	50,4	62,8

DISCUSSION

Raising the cyanide contents by a factor of 5 in *Arabidopsis* plants has neither an effect on the expression of *ST* and *CAS* mRNA and the respective protein contents nor on the ST and CAS enzyme activity. In general, cyanide does not induce the expression or enhance the activity of ST and CAS. Constitutive expression levels might be sufficient for the detoxification of cyanide. In non-cyanogenic plants cyanide is mainly formed in the last step of ethylene biosynthesis which takes place in the cytosol. However, both enzymes, ST and CAS, are localized in the mitochondria. Therefore one has to postulate a transport of cyanide into the mitochondria. In summary, the evidence for an involvement of ST and also CAS in cyanide detoxification as their only role in the metabolism is very low.

CHAPTER 5:

FURTHER INVESTIGATION ON THE ROLE OF THE PROLONGED LINKER SEQUENCE IN PLANT SULFURTRANSFERASES

Michael Bauer and Jutta Papenbrock

INTRODUCTION

Sulfurtransferases/rhodaneses are a group of enzymes widely distributed in plants, animals, and bacteria that catalyse the transfer of a sulfur atom from a donor molecule to a thiophilic acceptor substrate (Westley, 1973). Sulfurtransferases (STs) consist of two globular domains (N-terminal and C-terminal) of nearly identical size and conformation connected by a short linker sequence which is prolonged in ST sequences from plants (Fig. 1). The C-terminal domain contains the active site cysteine residue. In plant sequences the connecting linker sequence between both domains is quite long in comparison to sequences from prokaryotes and other eukaryotes. We would like to elucidate the role of the prolonged linker and each single domain of STs from *Arabidopsis thaliana*.

MATERIALS AND METHODS

The N-terminal and the C-terminal domains without and including the linker of ST1 from *Arabidopsis* (Acc. no. CAB64716) were expressed in *E. coli* with a 6xHis-tag at the N-terminus, purified by nickel-affinity chromatography and used for enzyme activity determinations. Enzyme assays were done as described using 3-mercaptopyruvate as sulfur donor and cyanide as sulfur acceptor (Papenbrock and Schmidt, 2000a).
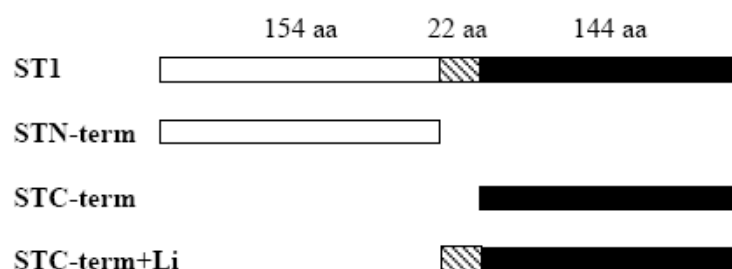

Fig. 1. The *Arabidopsis* ST1 protein consists of two domains, an N-terminal (STN-term) and a C-terminal domain (STC-term). Both are connected by a prolonged linker. Li, linker.

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RESULTS

The N-terminal domain of ST1 (STN-term) alone did not show enzyme activity at all whereas the activity of the C-terminal domain of ST1 (STC-term) without and including the linker sequence was very low but anyway well measurable (Fig. 2). To investigate the possibility of reconstitution the full activity compared with the activity of the complete recombinant ST1, increasing amounts of the STN-term were added to the STC-term without and including the linker. Increasing amounts of the STN-term protein resulted in increased enzyme activities for STC-term without and STC-term including the linker sequence. With regard to the specific activity of the whole ST1 enzyme the levels were much lower (Burow et al. 2002). Interestingly, the combination of the STN-term with the STC-term including the linker resulted in lower specific activities than the interaction with the STC-term without the linker. When thiosulfate was used as substrate neither the STC-term including nor the STC-term without the linker did show any activity.
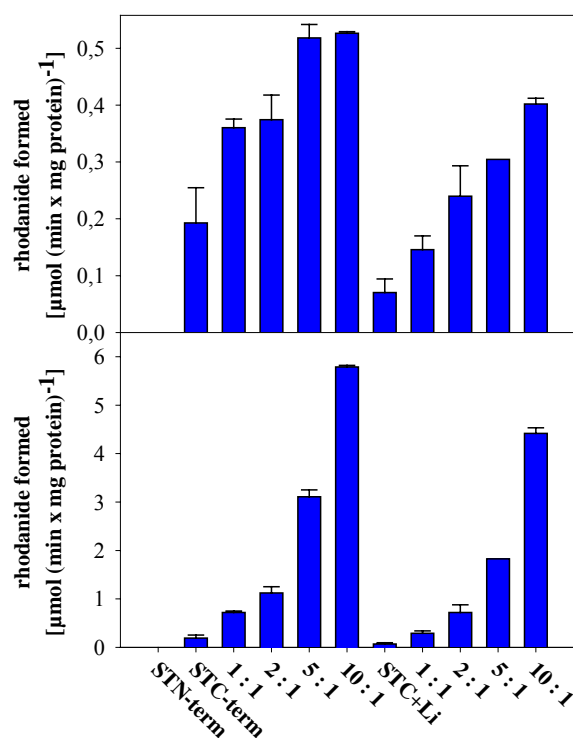
Fig. 2. The N-terminal domain was combined with the C-terminal domains without and including the linker in increasing mass-to-mass ratios and used for enzyme activity measurements as described. Sulfur donor: 3-mercaptopyruvate; sulfur acceptor: cyanide. (A) The specific enzyme activity was calculated on the basis of the total protein or (B) on the STC-term protein content.

DISCUSSION

The enzymatic activity of the *Arabidopsi*s ST1 resides in the C-terminal domain, which contains the active site cysteine residue, but is boosted by the N-terminal domain and the linker peptide in the full length enzyme. The presence of about two times more N-terminal protein molecules might protect the active site cysteine residue better than equal numbers of molecules. On account of this the role of the prolonged linker remains unclear because the specific activities were even lower in the combination experiments. Probably neither 3-mercaptopyruvate nor thiosulfate are metabolised in the plant organism by STs. A larger substrate might need the extended protective hydrophobic environment of the prolonged linker sequence in plants.

CHAPTER 6:

INTRACELLULAR LOCALIZATION OF *ARABIDOPSIS THALIANA* SULFURTRANSFERASES

Michael Bauer, Christof Dietrich, Katharina Nowak, Walter D. Sierralta, and Jutta Papenbrock

ABSTRACT

Sulfurtransferases (Strs) comprise a group of enzymes widely distributed in archaea, eubacteria, and eukaryota which catalyze the transfer of a sulfur atom from suitable sulfur donors to nucleophilic sulfur acceptors. In all organisms analyzed to date, small gene families encoding Str proteins have been identified. The gene products were localized to different compartments of the cells. Our interest concerns the localization of Str proteins encoded in the nuclear genome of *Arabidopsis thaliana*. Computer-based prediction methods revealed localization in different compartments of the cell for six putative AtStrs. Several methods were used to determine the localization of the AtStr proteins experimentally. For AtStr1, a mitochondrial localization was demonstrated by immunodetection in the proteome of isolated mitochondria resolved by one- and two-dimensional gel electrophoresis and subsequent blotting. The respective mature AtStr1 protein was identified by mass spectrometry sequencing. The same result was obtained by transient expression of fusion constructs with the green fluorescent protein (GFP) in *Arabidopsis* protoplasts, whereas AtStr2 was exclusively localized to the cytoplasm by this method. Three members of the single-domain AtStr were localized in the chloroplasts as demonstrated by transient expression of GFP fusions in protoplasts and stomata, whereas the single-domain AtStr18 was shown to be cytoplasmic. The remarkable subcellular distribution of AtStr15 was additionally analyzed by transmission electron immunomicroscopy using a monospecific antibody against GFP, indicating an attachment to the thylakoid membrane. The knowledge of the intracellular localization of the members of this multi-protein family will help elucidate their specific functions in the organism.

INTRODUCTION

All members in the sulfurtransferase/rhodanese (Str) protein family in archaea, eubacteria, and eukaryota are unified by characteristic well-defined sequence domains (Bordo and Bork, 2002). These domains are found as tandem repeats, with the C-terminal domain containing the active site cysteine residue, as single-domain proteins or as members of multi-domain proteins (Bordo and Bork, 2002). The 18 proteins identified in *Arabidopsis* which contain at least one Str signature were classified into six groups on the basis of their sequence homology (Bauer and Papenbrock, 2002; http://arabidopsis.org/info/genefamily/STR_genefamily.html). Group I consists of two Str proteins with two-domain; the five proteins in group VI contain only the C-terminal Str signature and thus possess similarity to the single-domain Str from bacteria.

Strs catalyze the transfer of a sulfur atom from suitable sulfur donors to a nucleophilic acceptor. Specific biological roles for most members of this superfamily have not been established (Spallarossa et al., 2001). Proposed roles include cyanide detoxification (Vennesland et al., 1982), involvement in sulfate assimilation (Donadio et al., 1990), and mobilization of sulfur for iron-sulfur cluster biosynthesis or repair (Bonomi et al., 1977). The most studied and best characterized Str is bovine rhodanese (thiosulfate:cyanide Str, EC 2.8.1.1) which catalyzes *in vitro*, the transfer of a sulfane sulfur atom from thiosulfate to cyanide, leading to the formation of sulfite and thiocyanate (Westley, 1973).

Strs have been identified in different compartments in living organisms. In *Escherichia coli,* seven Str proteins were identified; a single-domain Str, GlpE, is a cytoplasmic protein, whereas at least one two-domain Str was localized in the periplasm (Ray et al., 2000). In the cyanobacterium *Synechococcus* sp. strain PCC 7942, a rhodanese-like protein was localized to the periplasmic space and was suggested to play a role in the transport of specific sulfur compounds (Laudenbach et al., 1991). In mammalia two different Str enzymes with 3-mercaptopyruvate and thiosulfate specific activities have been identified which are characterized by different K_m values for both substrates. The 3-mercaptopyruvate Str protein was localized by immunogold-labelling and Western blot analysis to the cytoplasm and the mitochondria, whereas thiosulfate Str was detected exclusively in mitochondria and mainly in liver cells. 3-mercaptopyruvate Str might detoxify cyanide in the cytoplasm and in the mitochondria

both Str enzymes would protect cytochrome c oxidase effectively (Nagahara et al., 1995; Nagahara et al., 1999). The intracellular localization of Str1 and Str2 of *Arabidopsis* has been investigated previously (Hatzfeld and Saito, 2000; Nakamura et al., 2000; Papenbrock and Schmidt, 2000a), however the localization results obtained by fusion with the green fluorescent protein (GFP) were ambiguous. Recently, the single-domain Str protein, Ntdin from tobacco, a homologue of the AtStr15 protein from *Arabidopsis* with 56.8% identity, was localized in the chloroplasts by transient expression of GFP fusions (Yang et al., 2003), supporting the early report of the Str/rhodanese presence in the chloroplasts (Tomati et al., 1972).

Our aim of the research in this paper is the functional analysis of all members of the nuclear encoded Str multi-protein family in *Arabidopsis*. Knowledge of their intracellular localization is a prerequisite for the determination of their metabolic roles in multi-cellular organisms. Computer-based prediction programs are not yet sufficiently reliable and are only indicative of the *in vivo* localization of proteins. For exclusively immuno-based localization methods, many monospecific antibodies which are also difficult to produce against highly similar proteins, would be required. The mitochondrial localization of AtStr1 was demonstrated by several different methods leading to the same conclusions. The transient expression of GFP fusion proteins in *Arabidopsis* protoplasts was established as a reliable method for the determination of the intracellular localization as confirmed by a number of controls.

MATERIALS AND METHODS

Plant material

Arabidopsis thaliana Heynh. (ecotype C24) and *Nicotiana tabacum* L. (cv. Samsun NN) plants were grown on soil in the greenhouse in a 16 h light/8 h dark cycle 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 m^{-2} s^{-1} (sodium vapor lamps, SON-T Agro 400, Philips). *Arabidopsis* (ecotype C24) suspension cultures were established and grown as described by May and Leaver (1993).

DNA cloning techniques

The origins of templates and conditions for the amplification of cDNAs encoding AtStr1 (At1g79230), AtStr2 (At1g16460), AtStr15 (At4g35770), AtStr16 (At5g66040), and AtStr18 (At5g66170) have been described previously (Papenbrock and Schmidt, 2000a, b; Bauer and Papenbrock, 2002). For the amplification of a cDNA sequence encoding AtStr14 (At4g27700), the full length EST clone RAFL05-17-G02 was obtained from RIKEN BioResource Center, Tsukuba, Japan. All primer pairs used for the construction of fusion clones with the green fluorescent protein (GFP) are listed in Table 1.

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, followed by 28 PCR cycles of 45 s at 94°C, 45 s at 55°C, and 45 s at 72°C each. 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 to obtain either GFP fusions with the 5' end of the GFP coding sequence (pGFP-N) or with the 3' end (pGFP-C) and were introduced into the *E. coli* strain XL1-blue. The gene-cassettes were driven by the CaMV-35S promoter with a double enhancer and the polyA-tail from CaMV-35S. Clones were sequenced for conformation of the insert using specific primers GFPforward 5'-CTG GAG TTC GTG ACC GCC GCC GG-3' or GFPreverse 5'-GCT TGC CGT AGG TGG CAT CGC CCT-

3' (MWG Biotech, Ebersberg, Germany). The expressed Str proteins were either fused with the N-terminus of the GFP (pGFP-N) or with the C-terminus of the GFP (pGFP-C).

Table 1. Overview of the GFP fusion clones produced. The name of the fusion construct, the length of the PCR amplified DNA fragment, the primer pairs designed with the respective restriction sites and the respective vector used for the fusions 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 site	Fusion vector
AtStr1woPS	963	P194 5'-cca tgg ctt cta ctg gag ttg a-3'	*Nco*I	pGFP-N
		P195 5'-cca gat ctt gaa gaa gat tca ac-3'	*Bgl*II	pGFP-C
AtStr1wPS	600	P192 5'-cca tgg cct cga ccc ttt tct-3'	*Nco*I	pGFP-N
		P193 5'-gga tcc cca tct tgg tag acc t-3'	*Bam*HI	
AtStr2woPS	954	P153 5'-cca tgg ctt ctt ctg gat ctg a-3'	*Nco*I	pGFP-N
		P154 5'-gga tcc tga aga aga acc cac t-3'	*Bam*HI	pGFP-C
AtStr2wPS	1098	P155 5'-cca tgg cac gag gag aat ctg-3'	*Nco*I	pGFP-N
		P154 5'-gga tcc tga aga aga acc cac t-3'	*Bam*HI	
AtStr14	711	P138 5'-cca tgg ctt cac tta ctt caa-3'	*Nco*I	pGFP-N
		P189 5'-cag atc tgt ctt ctt caa ttg ttt c-3'	*Bgl*II	
AtStr15	546	P132 5'-cca tgg aaa cca ctg ctt tta ac-3'	*Nco*I	pGPF-N
		P150 5'-aga tct ctc ttc tac cgg cag c-3'	*Bgl*II	pGFP-C
AtStr16	360	P134 5'-cca tgg acg agg aga gca gag t-3'	*Nco*I	pGFP-N
		P151 5'-aga tct ttg aag aag aag gag acg-3'	*Bgl*II	
AtStr18	408	P136 5'-cca tgg ctc aat caa tct cct cc-3'	*Nco*I	pGFP-N
		P152 5'-aga tct att agc aga tgg ctc ctc-3'	*Bgl*II	pGFP-C

Transient expression of GFP fusion constructs in N. tabacum *leaves*

Plasmid DNA from the constructs described above was column-purified (Plasmid Midi Kit, Qiagen, Hilden, Germany). Gold particles (1.0 µm gold microcarrier, Bio-Rad Laboratories GmbH, Munich, Germany) were covered with plasmid DNA as follows (protocol for 6 repetitions): 3 mg gold was washed in pure 70% ethanol, centrifuged for 1 min at 1,925g with braking. The gold pellet was resuspended in 50 µl H$_2$O and again centrifuged at 47g without braking. The pellet was resuspended in 50 µl 50% glycerol by mixing and ultrasonification for 10 sec. After adding 7 µg plasmid DNA dissolved in 7 µl H$_2$O and incubation for 5 min on ice, 50 µl 2.5 M CaCl$_2$ and 20 µl cold 1 M spermidine were added. The suspension was centrifuged for 15 sec at 47g and the pellet was resuspended in 100% ethanol by mixing and ultrasonification. For the bombardment, 5 µl of the DNA-covered gold particles were distributed on a macrocarrier/flying disk (Bio-Rad). The rupture disks (Bio-Rad) used could withstand 900 pounds per square inch. Pieces (4 x 4 cm) of fully expanded *N. tabacum* leaves were cut from 4-week-old plants by avoiding the middle rib. The leaf pieces were placed up-side-down on moistened filter paper in petri dishes (9 cm diameter). The particle delivery system was used according to the manufacturer's instructions (Bio-Rad). The treated leaf pieces were incubated for about 16 h at room temperature. Then the epidermis of the tobacco leaves was removed and placed in 0.3 M sorbitol on a glass slide for microscopic analysis.

Transient expression of GFP fusion constructs in Arabidopsis *protoplasts*

The younger rosette leaves of 3-week-old *Arabidopsis* plants were used for the preparation of protoplasts according to Damm et al. (1989), Sheen (1995), and Abel & Theologis (1998) with some modifications. About 40 leaves were cut in 1 mm strips with sharp razor blades and put in 6 ml of medium I (1% [w/v] cellulase Onozuka R-10, 0.25% [w/v] macerozyme R-10 [Yakult Honsha, Tokyo, Japan], 0.4 M mannitol, 20 mM KCl, 20 mM MES/KOH, pH 5.7, 10 mM CaCl$_2$, 5 mM β-mercaptoethanol, 0.1% [w/v] bovine serum albumine [BSA]). After application of a vacuum for 15 min, the leaves were incubated on a shaker (40 rpm) for 60 min at room temperature. The

suspension was filtered through a 75 µm-pore nylon net, distributed on 4 2 ml-tubes and centrifuged for 2 min at 95g and 4°C. The pellets were washed twice with 500 µl of medium II (154 mM NaCl, 125 mM CaCl$_2$, 5 mM KCl, 2 mM MES/KOH, pH 5.7) and finally incubated for 30 min on ice in medium II. After centrifugation for 2 min at 95g and 4°C the pellets were carefully resuspended in 150 µl of medium III (0.4 M mannitol, 15 mM MgCl$_2$, 4 mM MES/KOH, pH 5.7). For the transformation, 100 µl of the protoplast suspension was mixed carefully with 15 µg column-purified plasmid DNA and 110 µl medium IV (4 g polyethylene glycol [PEG] 4,000, 3 ml H$_2$O, 2.5 ml 0.8 M mannitol, 1 ml 1 M CaCl$_2$), and incubated for 30 min at 23°C. To remove the PEG, the solution was washed with 1 ml of medium II, centrifuged for 2 min at 95g and 4°C. The pellet was resuspended in 50 µl of medium II and incubated overnight at room temperature.

In some experiments MitoTracker Orange CMTMRos (Molecular Probes, Eugene, OR) was used to stain mitochondria. The dye was dissolved in DMSO to 1 mM and was incubated in a final concentration of 0.5 µM with the protoplast suspension for 15 min.

Microscopic analysis

The transiently-transformed cells and protoplasts were analyzed with an Axioskop microscope (Carl Zeiss, Jena, Germany). The GFP fluorescence was collected with the band pass filter (BP 450-490) for excitation and with the long pass filter (LP 520) for emission. The generation of transmission micrographs for visualization of non-fluorescent protoplast structures was achieved using the manufacturer´s filter settings. Confocal imaging of transformed protoplasts and stained mitochondria was performed with the True Confocal Scanner (Leica, TCS SP2, Solms, Germany). Specimens were examined using Leica 20x and 63x water immersion objectives. GFP fluorescence was excited with the argon laser (488 nm) and detected at 515 nm to 520 nm. MitoTracker Orange CMTMRos was excited with the green helium neon laser (543 nm) and detected at 575 nm to 585 nm. Far-red autofluorescence of chlorophyll was detected at 650 nm to 670 nm. All images were edited with Corel Photo Paint 10.

Localization studies by immunogold electron microscopy

The suspension of protoplasts was centrifuged at 375g in an Eppendorf microtube; the sediment was layered with 10 ul 1.5% gelatine in medium II and was then cooled on ice. After solidification of the gelatine, the samples were covered with 1 ml of fixative, consisting of 4% (w/v) freshly de-polymerized paraformaldehyde prepared in medium II and containing 0.5% (w/v) glutaraldehyde. After fixation for 30 min at 4ºC, the samples were washed with medium II, dehydrated at low temperature with graded alcohols, and embedded in LR-Gold as described previously (Sierralta, 2001). From the embedded tissues, ultrathin sections (70 nm thickness) were cut with a Reichert-Jung ultramicrotome; the sections were collected on Formvar-coated 200 mesh gold grids and immediately incubated as described (Sierralta et al., 1995). Briefly, after quenching and blocking, the ultrathin sections were incubated for 2 h at room temperature with rabbit anti-GFP (Molecular Probes, Eugene, OR) and thoroughly washed. The bound IgGs were tagged with goat anti-rabbit IgG labelled with 15 nm gold particles (British BioCell, Cardiff, UK). Finally, the sections were lightly stained with 5% aqueous uranyl acetate and Reynolds lead citrate. Immunolabelling controls included omission of the primary antibody or incubation of the sections with serum from a non-immunized rabbit. Sections were viewed with a Philips CM100 electron microscope at 80 kV, photographed on Kodak 4489 EM film, and printed on Agfa photographic paper. The digital images are from scans of the photomicrographs. A statistical analysis of the immunolabelling results was done according to established criteria (Griffith, 1993). Data were collected from 20 fields analyzed in 5 sections from 2 blocks of protoplasts from transiently-transformed leaves. The primary magnification used was 18,000x.

Organelle isolation, SDS-PAGE, and Western blotting

Mitochondria were isolated from *Arabidopsis* suspension cultures as described by Kruft et al. (2001). Chloroplasts were isolated from 4-week-old *Arabidopsis* plants according to Jensen and Bassham (1966). Total protein extracts from *Arabidopsis* plants were obtained as described previously (Papenbrock and Schmidt, 2000b). Aliquots of approximately 10 µg protein determined following the method of Stoscheck (1990)

were analyzed by one-dimensional SDS-PAGE (Laemmli, 1970). The mitochondrial proteome was separated by two-dimensional gel electrophoresis (50 μg protein, IEF range pH 3-10) (Kruft et al., 2001). The resulting gels were blotted onto nitrocellulose membranes (Sambrook et al., 1989). For immunodetection a monospecific antibody directed against the recombinant AtStr1 protein was used (Papenbrock and Schmidt, 2000a). The protein spots corresponding to the immunostained proteins were localized on a Coomassie-stained (Neuhoff et al., 1985) two-dimensional gel loaded with 1 mg mitochondrial protein and analyzed by mass spectrometry.

Miscellaneous

The analyses of DNA and amino acid sequences were performed with the programs EditSeq and MapDraw in DNASTAR (Madison, WI, USA). For the computer-based prediction of the protein localization different programs were applied (mainly PSORT, SignalP V2.0, TargetP, and further programs in http://www.expasy.ch/tools). After the transient transformation methods had been established, the transformations were performed with each clone at least five times resulting always in the same intracellular localization.

RESULTS

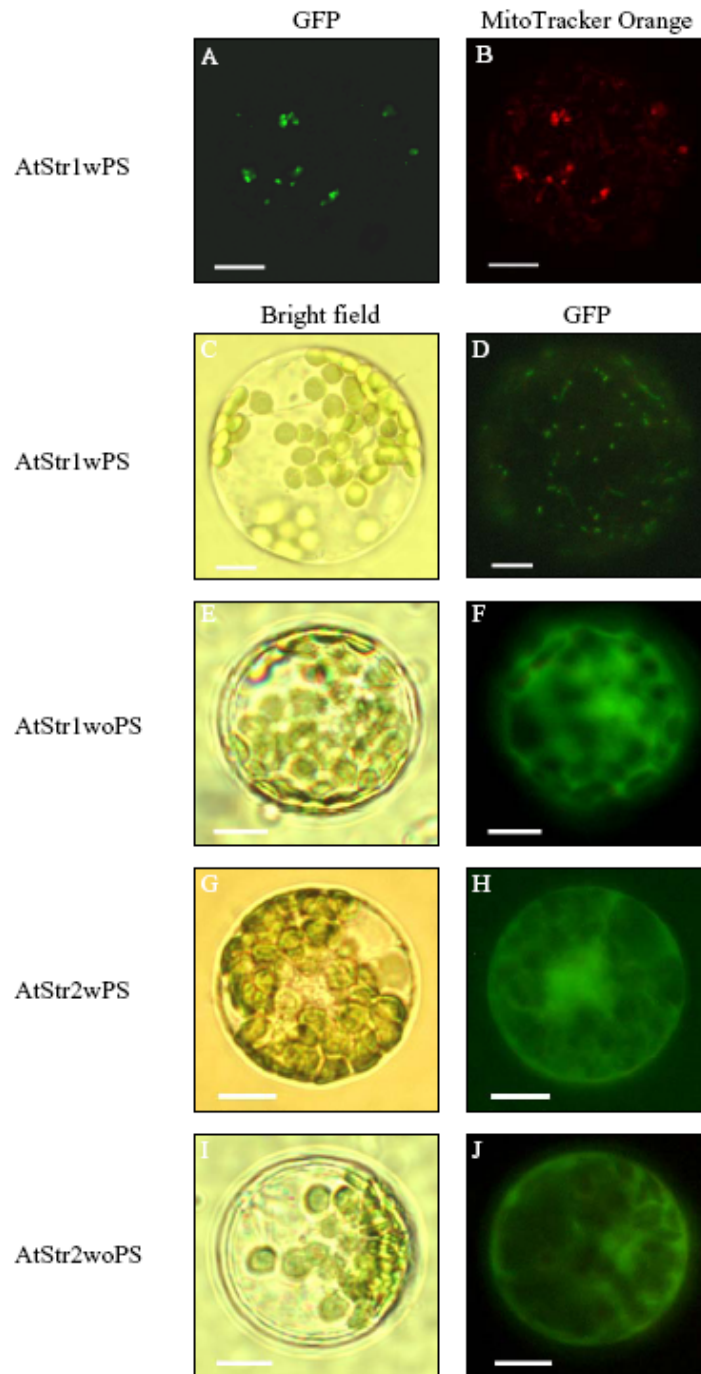*Intracellular localization of AtStr1 in mitochondria*

Arabidopsis protoplasts were transformed using a transient expression system with *AtStr1* and *AtStr2* including (wPS) or excluding (woPS) their putative targeting peptide sequences (Fig. 1). Co-localization of the AtStr1wPS/pGFP-N fluorescence with MitoTracker Orange fluorescence, a dye that is specifically enriched in mitochondria, in the same protoplasts using a confocal laser scanning microscope (CLSM), resulted in the same picture. A second control for mitochondrial intracellular localization was used: the targeting peptide of the mitochondrial protein, serine hydroxymethyltransferase (SHMT), was fused to the GFP protein resulting in the same image as transient transformation with the AtStr1 fusion construct (data not shown). These results indicated import of the AtStr1 protein into the mitochondria (Fig. 1A, B). The same localization results were obtained with a fluorescence microscope (Fig. 1D). The corresponding bright field picture visualizes the protoplast's cell membrane, demonstrates the intactness of the protoplasts, and the position of the chloroplasts (Fig. 1C). *AtStr1* expressed without its putative targeting peptide sequence remained in the cytoplasm (Fig. 1F). The fusion construct of *AtStr1* with the targeting peptide sequence and the GFP-encoding cDNA sequence at the 5' end (AtStr1wPS/pGFP-C) resulted in a fluorescence image identical to the transformed pGFP-C vector alone (data not shown). This indicates that the N-terminal targeting peptide is recognized by the import machinery of the mitochondria.

AtStr2 protein was described to be cytoplasmic (Hatzfeld and Saito, 2000; Nakamura et al., 2000). However, the 5' end of the *AtStr2* sequence was possibly not correctly determined, since the EST clone ATTS6033 obtained from the Arabidopsis Biological Resource Center, Ohio State University, contained a putative targeting peptide of 48 amino acids (Table 2) (Papenbrock and Schmidt, 2000b). Therefore protoplasts were transformed with the respective constructs including or excluding the 5' extension (AtStr2wPS/pGFP-N, AtStr2wPS/pGFP-C, and AtStr2woPS/pGFP-N). All AtStr2 fusion proteins remained in the cytoplasm. Obviously, the additional DNA at the 5' end

did not encode a putative targeting peptide (Fig. 1G-J) (AtStr2wPS/pGFP-C localization data not shown).

To demonstrate the reliability of localization studies using GFP fusion proteins, methods based on antibody specificity were applied. Mitochondria were purified from *Arabidopsis* cell cultures, chloroplasts were enriched from green *Arabidopsis* plants, and total protein extracts were prepared from green *Arabidopsis* plants. In the Western blot analysis, a protein of about 35 kD was detected in the mitochondrial fraction, corresponding to the predicted size of the mature AtStr1 protein (35.6 kD) (Fig. 2A). In total extracts, a protein of about 43 kD was found close to the predicted size of the AtStr1 protein including the targeting peptide (41.9 kD).

The proteome of mitochondria purified from *Arabidopsis* cell cultures was separated by two-dimensional gel electrophoresis (50 μg protein) and was subject to Western blot analysis (Fig. 2B, top). In comparison to the Western blot of the one-dimensional gel, the development of the color reaction was prolonged to detect as many cross-reacting proteins as possible without obtaining too much background. Two protein spots, a major and a minor, were identified in the immunoreaction. The molecular mass and the isoelectric point of the major spot are in agreement with the predicted values (35.6 kD/pI 5.01) of the mature AtStr1 protein. In parallel, the mitochondrial proteome (1 mg protein) was separated under the same conditions, and the gel was Coomassie-stained. The protein spots corresponding to the proteins recognized by the AtStr1-specific antibody are marked by arrows (Fig. 2B, bottom). In the MALDI/TOF-MS and ESI-MS/MS analysis, both the major and the minor protein spots were identified as AtStr1 proteins. The difference in the molecular mass may be explained by a posttranslational modification, such as a phosphorylation. The nature of the modification is still unknown. The sequence of the N-terminal peptide of the mature AtStr1 could be determined and the computer-predicted cleavage site was confirmed experimentally. The total length of targeting peptide was calculated to 58 amino acids, the bar marks the cleavage site: MASTL….WARRA_MASTG.

Fig. 1. Subcellular localization of AtStr1 and AtStr2 GFP fusion constructs with and without signal peptide. (A) *Arabidopsis* protoplasts were transformed with *AtStr1* including its pre-sequence (AtStr1wPS). Fluorescence images of the protoplasts were taken using a confocal laser scanning microscope. The GFP fluorescence was excited with the argon laser (488 nm) and detected at 515 nm to 520 nm. (B) The same protoplast suspension was additionally stained with MitoTracker Orange CMTMRos. The fluorescence was excited with the green helium neon laser (543 nm) and detected at 575 nm to 585 nm. (C to J) *Arabidopsis* protoplasts were transiently transformed with AtStr1 and AtStr2 constructs with and without the pre-sequences (AtStr1wPS/AtStr1woPS and AtStr2wPS/AtStr2woPS, respectively). Bright field images shown in C, E, G, and I were made to visualize the protoplast's cell membrane and the chloroplasts. Fluorescence images of the protoplasts shown in D, F, H, and J were taken using an Axioskop microscope with filter sets optimal for GFP fluorescence (BP 450-490/LP 520). All scale bars represent 10 μm.
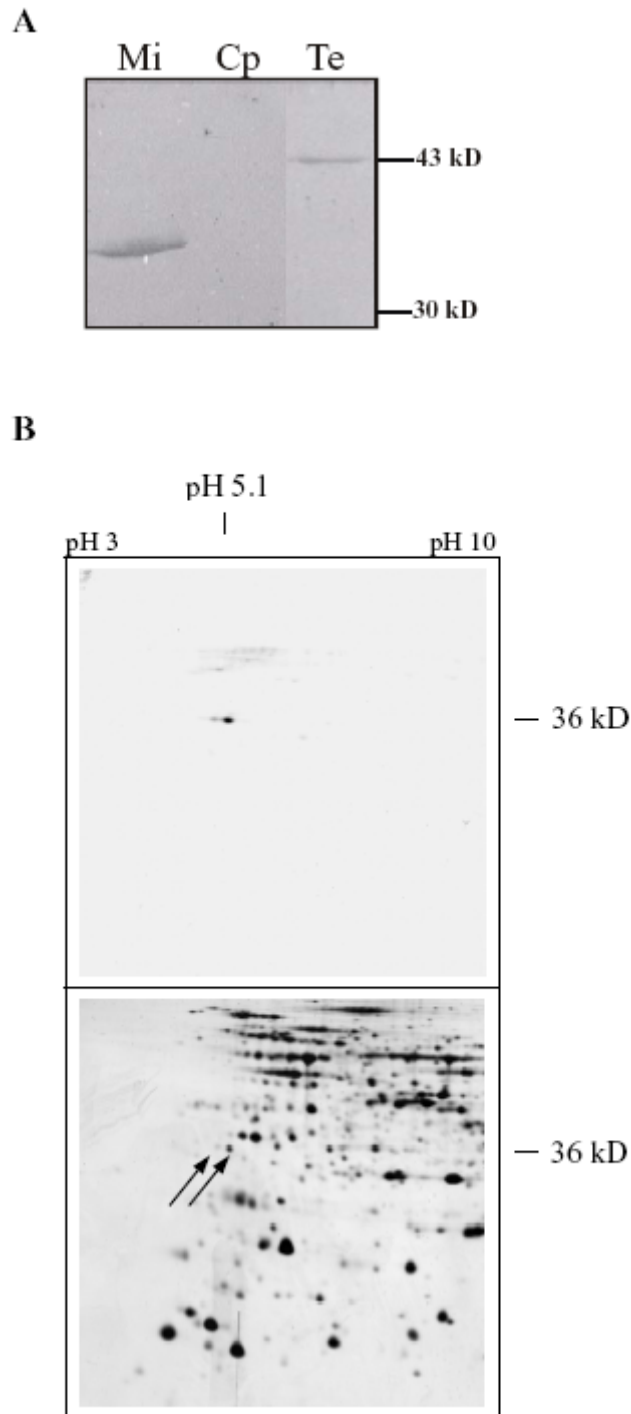
Fig. 2. Protein gel electrophoresis and subsequent Western blot analysis of total and organellar extracts. (A) Mitochondria (Mi) were purified from *Arabidopsis* cell cultures, chloroplasts (Cp) were isolated from green *Arabidopsis* plants, and total soluble protein extracts (Te) were also obtained from green *Arabidopsis* plants. The proteins were separated by one-dimensional gel electrophoresis, blotted, and the membranes were incubated with an antibody directed against the AtStr1 protein. (B) Mitochondria were purified as described above. Their proteome was separated by two-dimensional gel electrophoresis and analyzed by Western blot analysis as described above (top). The corresponding protein spots marked by arrows were localized on a Coomassie-stained gel that was run in parallel. The spots were cut and the proteins were analyzed by mass spectrometry.

Three single-domain AtStrs are localized in chloroplasts and one is localized in the cytoplasm

Group VI of the AtStr family contains five members which have single-domains (Table 2). To clarify their function, as many features as possible of each single AtStr should be determined. Intracellular localization of four of the single-domain AtStrs was analyzed. To date, no EST clone of AtStr17 has been isolated (http://www.uni-frankfurt.de/fb15/botanik/mcb/AFGN/papen.htm). RT-PCR using different mRNA-pools and screening of cDNA libraries with sequence specific primers was not successful, indicating that the gene encoding AtStr17 may be a pseudogene.

Nicotiana tabacum leaf cells were transformed with the transient expression system with the fusion constructs AtStr14/pGFP-N and AtStr14/pGFP-C or AtStr18/pGFP-N and AtStr18/pGFP-C using the ballistic method of particle gun bombardment. Transformed tobacco leaf epidermal and guard cells were analyzed by fluorescence microscopy after overnight incubation (Fig. 3). AtStr14 was transported into plastids as seen in the images of the GFP fluorescence in plastids of epidermal and guard cells (Fig. 3B, C). The bright field image of an epidermal *N. tabacum* cell shows the cell dimensions (Fig. 3A). In pre-tests, leaves of *Arabidopsis* were also transformed by particle bombardment and led to the same localization of the *Arabidopsis* proteins as the heterologous transformation of *N. tabacum* leaves. Controls using a GFP fusion construct containing the transit peptide of RubisCO, revealed comparable images as shown in Fig. 3B and 3C (data not shown). Figure 3D and E show the same guard cell transformed with the AtStr18/pGFP-N construct: GFP fluorescence was detected in the cytoplasm.

To examine targeting of four out of five of the single-domain AtStr, fusion constructs of AtStr14, AtStr15, AtStr16, and AtStr18 with pGFP-N or pGFP-C were introduced into *Arabidopsis* protoplasts, incubated overnight at room temperature, and visualized by fluorescence microscopy (Fig. 4). Bright field images were taken to visualize the protoplast's cell membrane and chloroplasts. AtStr14, AtStr15, and AtStr16 were localized in chloroplasts whereas AtStr18 remained in the cytoplasm. The choice of the transformation method, either particle bombardment or PEG-mediated protoplast transformation, did not affect the localization results as shown by Figures 3 and 4.

Table 2. Localization of sulfurtransferases. The protein name, gene identification, protein description, and the number of amino acids for six selected sulfurtransferases are summarized. The programs PSORT and TargetP for the localization prediction, and the program SignalP V2.0 for the cleavage site prediction were used (http://www.expasy.ch/tools). Numbers in brackets give the certainty of prediction in PSORT, for the TargetP program the respective probabilities are given. The results of the experiments done are summarized. Abbreviations: aa, amino acids; ass., associated; chloroplast; ID, identification; m, mitochondrial; no., number; n.d., not determined; SP, secretory pathway; TP, transit peptide.

Gene ID	Description	No. aa	Localization prediction		Experimental
		TP/total	PSORT	TargetP	
At1g79230	thiosulfate Str	58/379	mitochondrial matrix space (0.814) microbody (peroxisome) (0.579)	cTP - 0.666 mTP - 0.319 SP - 0.007	mitochondrion
At1g16460	thiosulfate Str	48/366	chloroplast stroma (0.607) cytoplasm (0.450)	cTP - 0.233 mTP - 0.040 SP - 0.165	cytoplasm
At4g27700	hypothetical protein	34/237	chloroplast stroma (0.950) chloroplast thylakoid membrane (0.800)	cTP - 0.816 mTP - 0.258 SP - 0.025	chloroplast
At4g35770	senescence-ass. protein	63/182	chloroplast stroma (0.837) mitochondrial matrix space (0.598)	cTP - 0.822 mTP - 0.067 SP - 0.008	chloroplast
At5g66040	senescence-ass. protein	79*/120	cytoplasm (0.450) mitochondrial matrix space (0.360)	cTP - 0.082 mTP - 0.108 SP - 0.136	chloroplast
At2g17850	senescence-ass. protein	28*/150	nucleus (0.760) microbody (peroxisome) (0.364)	cTP - 0.033 mTP - 0.074 SP - 0.136	n.d.
At5g66170	sen.-associated protein	10*/136	chloroplast stroma (0.889) chloroplast thylakoid membrane (0.555)	cTP - 0.237 mTP - 0.064 SP - 0.120	cytoplasm
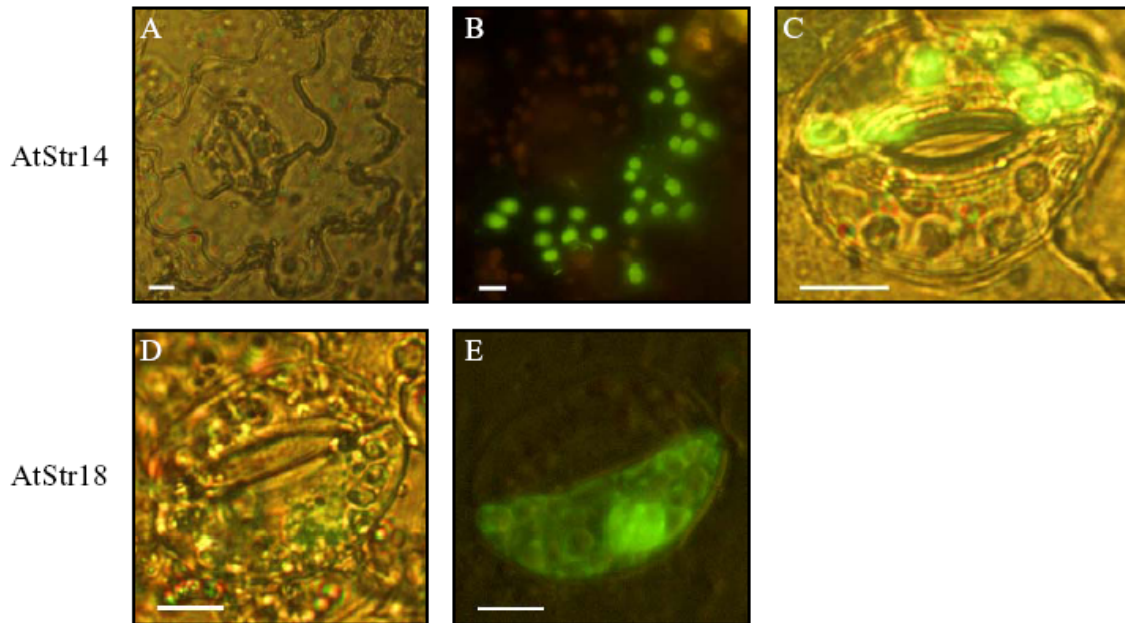
Fig. 3. Localization of the single-domain sulfurtransferases AtStr14 and AtStr18 in tobacco leaf epidermal and guard cells. Fusion constructs of AtStr14 and AtStr18 with the GFP encoding sequence were transformed in *N. tabacum* leaf cells by particle gun bombardment and were analyzed by fluorescence microscopy after overnight incubation. (A) Bright field image of an epidermal *N. tabacum* cell. (B) In the same cell shown in A the fluorescence of the AtStr14/pGFP-N fusion protein was collected with the band pass filter (BP 450-490) for excitation and with the long pass filter (filter LP 520) for emission. (C) Single merged image of a guard cell for AtStr14. (D) Bright field image of a guard cell. (E) In the same guard cell shown in D the fluorescence was collected as described in B. All scale bars represent 10 μm.

The AtStr15 protein is associated with the thylakoid membrane

In contrast to AtStr14 and AtStr16, which were evenly distributed in the chloroplasts and which is indicative for soluble proteins, the intracellular localization of the AtStr15 protein was unusual (Fig. 4). It was not possible to unambiguously determine whether the protein was outside of the chloroplast, on the chloroplast envelope or attached to the thylakoid membranes inside the chloroplast. Therefore we investigated its localization in more detail. Protoplasts were transiently-transformed with AtStr15/pGFP-N and analyzed with the CLSM (Fig. 5). In Figure 5C, the merged image of GFP fluorescence and chlorophyll autofluorescence of the same protoplast is shown. In Figure 5D, coordinating lines show the localization of AtStr15 (shown on the sidelines) in more detail. A higher magnification of the same protoplast enabled visualization of the thylakoid membranes and indicated an association of AtStr15 with the thylakoid

membrane (Fig. 5E, F). However, the limited resolution of the CLSM and the extensive emission of the GFP fluorescence made a final conclusion with respect to the subcellular localization of AtStr15 difficult.

Transmission electron immunomicroscopy was used to investigate the subcellular localization further. Protoplasts were transformed with AtStr15/pGFP-N using a transient expression system and then incubated with antibodies directed against GFP and the secondary antibody was labeled with gold particles. The transmission images show an association of AtStr15 protein with the thylakoid membranes, and to a smaller extent localization in the stroma of the chloroplasts (Fig. 6).

The statistical analysis of the density of gold labeling in the chloroplasts indicate an effective transformation. In 43 mitochondria only a total of 3 gold particles were found, in the profiles of the plasma membrane from 10 protoplasts just 4 gold particles were detected. In an area of 152 μm^{-2} of cytoplasm, 38 gold particles were counted. In 27 chloroplasts, with an area of 144 μm^{-2}, 234 gold particles were found. More than 74% of the gold label in chloroplast was associated with the thylakoid membranes, the remaining in stroma (Table 3). As controls for the specificity of the attachment, sections were incubated either in the absence of the GFP-specific antibody or with pre-immune serum. Less than 1 gold particle per 10 μm^{-2} was seen in these control sections. Only few areas around some vacuoles displayed 2 or 3 gold particles above this background. However, this compartment is known as having relatively high unspecific affinity.

Table 3. Summary of the data of the immunolabelling studies. Data was collected from 20 fields analyzed in 5 ultrathin sections from 2 blocks of transiently-transformed protoplasts according to Griffiths (1993). In sections incubated either in the absence of antibody or with pre-immune serum, less than 1 gold particle per 10 μm^{-2} was seen. The means ± standard deviations are given. gp, gold particles.

Organelle	Density of immunogold label (gp 10 μm^{-2})
Mitochondria	below detection limit
Plasma membrane	below detection limit
Cytoplasm	3.6 ± 2.1
Chloroplasts*	18.5 ± 6.0

*Over 74% of the label is associated with the thylakoid membranes.
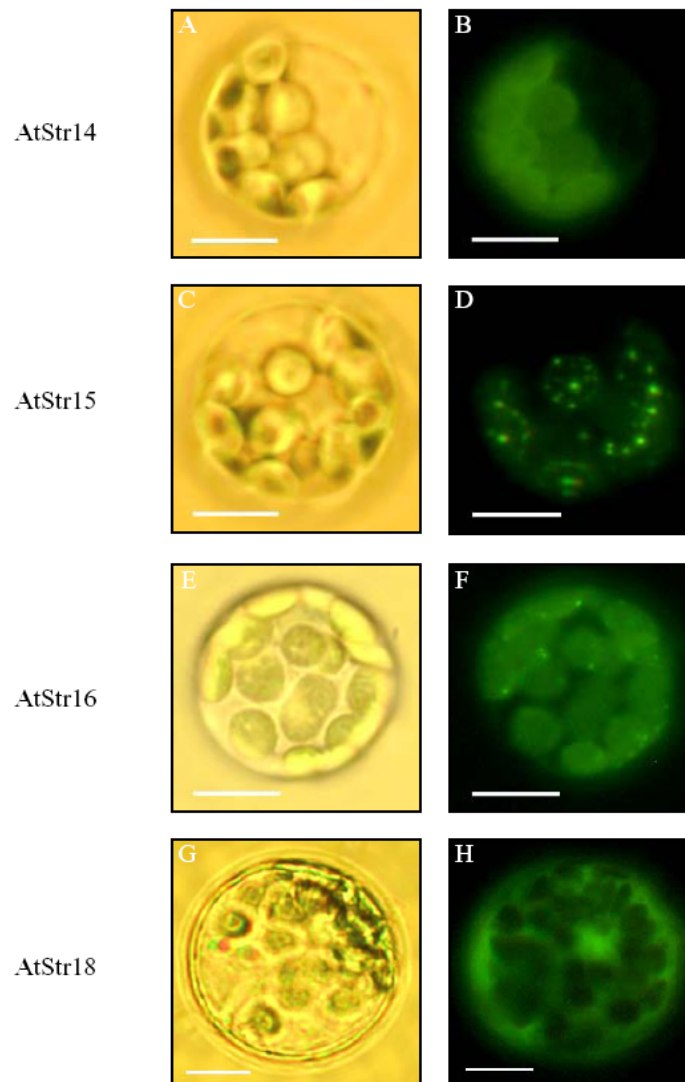
Fig. 4. Targeting analysis of all members of the AtStr group VI visualized by fluorescence microscopy. The fusion constructs of AtStr14, AtStr15, AtStr16, and AtStr18 with pGFP-N were introduced into *Arabidopsis* protoplasts. The protoplasts were incubated overnight at room temperature and then analyzed with an Axioskop microscope with filter sets optimal for GFP fluorescence (BP 450-490/LP 520). Bright field images (A, C, E, G) were made to visualize the protoplast's cell membrane and chloroplasts. Fluorescence images of the same protoplasts are shown in B, D, F and H.
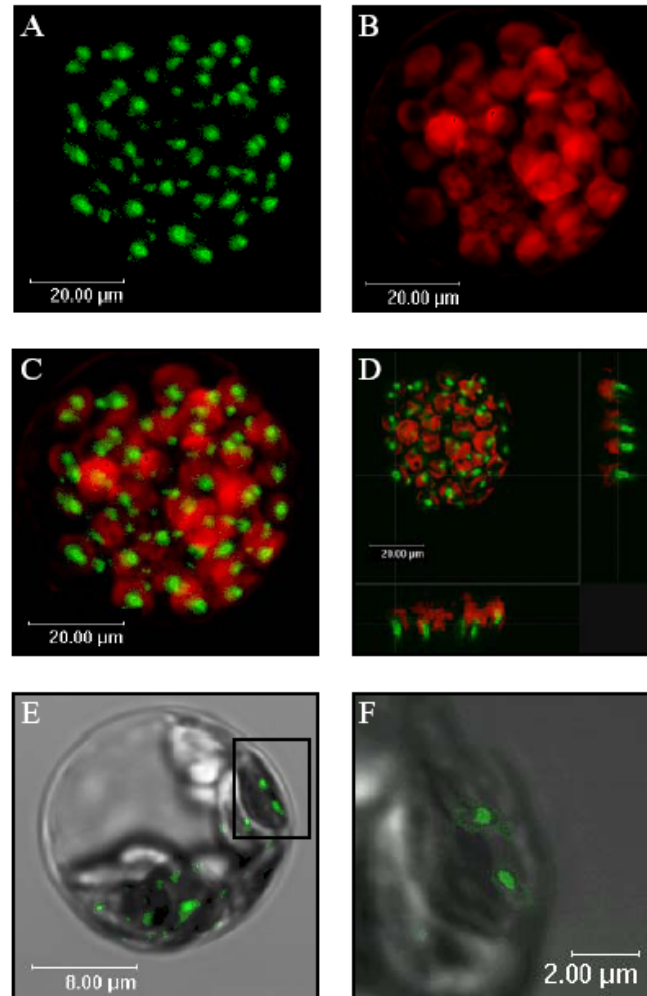
Fig. 5. Detailed analysis of the subcellular localization of AtStr15. A. thaliana protoplasts were transformed with the AtStr15/pGFP-N fusion construct. All images (A to F) were made with the True Confocal Scanner. GFP fluorescence was excited with an argon laser (488 nm) and detected at 515 nm to 520 nm. Chlorophyll autofluorescence (red) was detected simultaneously at 550 nm to 570 nm. (A) GFP fluorescence in a transformed protoplast. (B) chlorophyll fluorescence of the same protoplast as in A. (C) Merged image of A and B. (D) This figure represents the image shown in C having coordinating lines to show the localization of AtStr15 (shown on the sidelines). (E) GFP fluorescence of a single chloroplast merged with the respective bright field image. (F) Enlarged image of the inset of figure E. The sizes of the scale bars are given directly in the images.

Computer-based prediction of protein localization compared with experimental results

Several computer programs using different algorithms for the prediction of the intracellular localization of proteins were applied. The results of the prediction by computer programs are summarized in Table 2. For three out of six proteins, the localization predictions were in agreement with the experimental results (AtStr1, AtStr14, and AtStr15). For these proteins the probabilities given by both programs

PSORT and TargetP were very high. However, results in TargetP for AtStr1 suggested a dual-targeting into mitochondria and chloroplasts. The prediction probabilities for the other three proteins gave lower values, except for a chloroplast stroma localization of AtStr18 in PSORT. In the case of these lower certainties for the prediction, the experimental data gave completely different results.
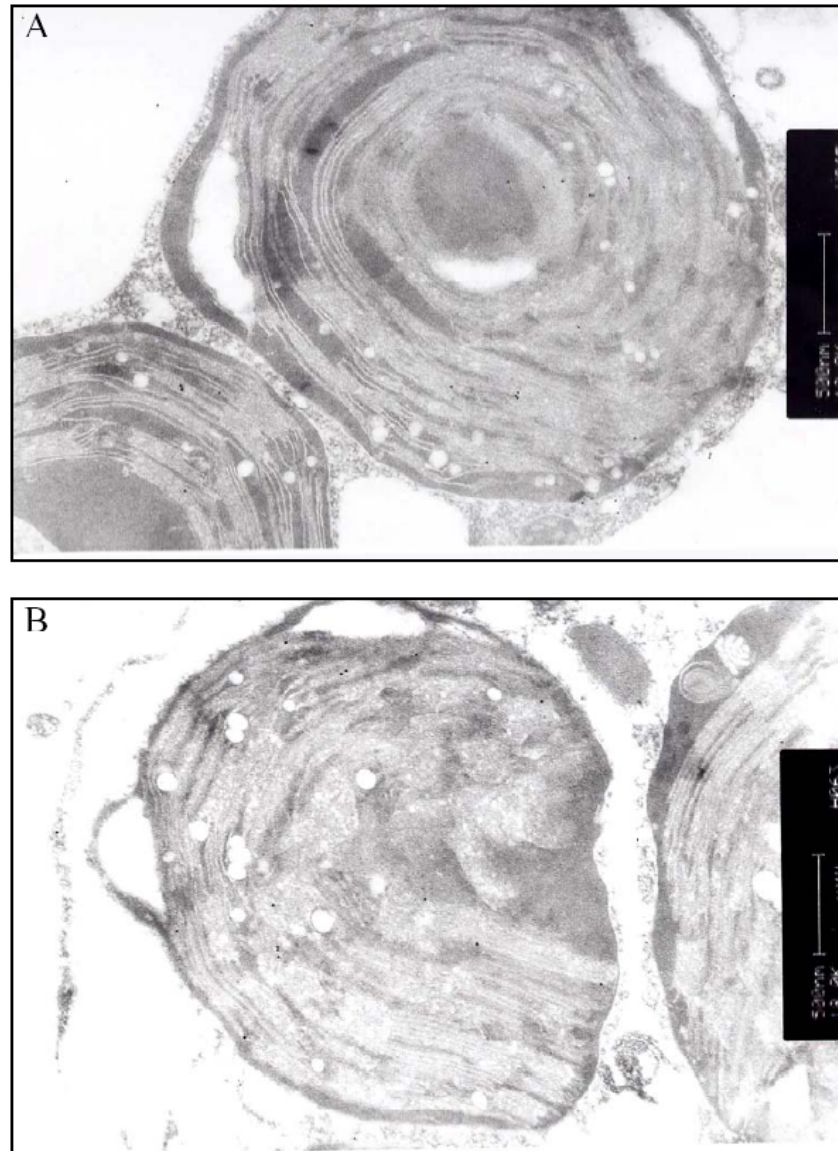
Fig. 6. Immunogold localization of GFP fusion protein in transiently transformed protoplasts by electron microscopy. (A) Chloroplast with numerous (>12) gold particles (i.e. GFP immunoreactivity) close to another chloroplast with and to a mitochondrion (below) without immunolabel. (B) Chloroplast at the left with about 12 gold particles at the thylakoid membrane. The mitochondrion at the right has no label. The chloroplast at the right contains only one label at the thylakoid membrane. The plasma membrane of the protoplast has one gold label.The scale bars represent 500 nm.

DISCUSSION

The pre-sequence of the nuclear encoded AtStr1 protein is cleaved after being transported into mitochondria

All Str proteins identified in *Arabidopsis* are nuclear-encoded proteins (Bauer and Papenbrock, 2002). Most of them were predicted to contain N-terminal domains potentially specifying intracellular targeting. The AtStr1 protein was shown previously to be localized in mitochondria by Western blot analysis (Nakamura et al., 2000; Papenbrock and Schmidt, 2000a) and GFP fusions (Hatzfeld and Saito, 2000; Nakamura et al., 2000). However, for the AtStr1 full-length fusion protein a dual-targeting was observed and also shorter versions of AtStr1 fused to GFP were found to target not only mitochondria, but also chloroplasts. On the basis of further localization experiments the authors concluded that the dual-targeting done with different GFP constructs did not reflect the actual subcellular distribution of AtStr1 in the plant cell (Nakamura et al., 2000). Very short fusion constructs might result in artifacts with respect to the intracellular localization (personal communication, Dr. R. Hänsch, Braunschweig). In our hands the fusion of the N-terminal extension of AtStr1 alone to GFP led to non-reproducible results, mainly indicating a cytoplasmic localization (data not shown). Therefore only the full-length proteins including their N-terminal or C-terminal extensions were used in this study. All AtStr sequences were ligated into both vectors, pGFP-N and pGFP-C, and were transiently-transformed. The GFP fluorescence of fusions with the pGFP-C vector always resulted in the same image as the pGFP-C and pGFP-N vector without any additional insert (data not shown). This fact indicates that none of the proteins investigated contained C-terminal targeting peptides for other compartments, such as the peroxisomes or the endoplasmatic reticulum (Emanuelsson and von Heijne, 2001).

The Western blot analyses of organelle and total protein extracts showed a mitochondrial localization of the mature AtStr1 protein. To identify the correct cleavage site, mitochondria were isolated from suspension cell cultures, the mitochondrial proteome was separated by two-dimensional gel electrophoresis and AtStr1 was detected by using a monospecific antibody against the protein. The determined size and isoelectric point corresponded with the computer prediction. The sequence of the mature protein's N-terminus and therefore the cleavage site matched the predictions. In this

particular case, all prerequisites for a successful application of this method were fulfilled. For a wide-spread use there are limitations such as the availability of monospecific antibodies. In addition, the isolation of pure organelles from cell cultures is a compromise since the correct localization of proteins might be affected by the intactness of the whole plant organism. However, the isolation of mitochondria (Hausmann et al., 2003) or other organelles from tissue of intact plants (Fukao et al., 2002) can be very difficult. Altogether the approach is rather laborious. Nevertheless for some proteins the method is successful.

Recently a high throughput analysis of the *Arabidopsis* mitochondrial proteome derived by liquid chromatography-tandem mass spectrometry was reported (Heazlewood et al., 2004). The AtStr1 protein and a second AtStr protein, AtStr3, which has not yet been characterized in detail (Bauer and Papenbrock, 2002) were identified as mitochondrial proteins by this approach. However, a number of false-positives for example several well-known plastidic proteins (e.g. At5g13630, At5g43780) were also found in the mitochondrial fraction, which reduces the reliability of this approach. Therefore the individual analysis of proteins with respect to the intracellular localization is still needed.

The single-domain AtStr are not localized in mitochondria

The transient transformation of *Arabidopsis* protoplasts using GFP fusion constructs was successfully established in our laboratory as described above. To confirm the translocation results obtained by this method, intact cells of *N. tabacum* and *Arabidopsis* leaves, respectively, were additionally transiently-transformed by particle bombardment with two single-domain AtStrs. It was reported that the translocation of proteins might be species-dependent. A homologous system was recommended, at least for import experiments of dual-targeting proteins (Lister et al., 2001). In our experiments, no difference could be observed between the use of tobacco and *Arabidopsis* leaves with respect to the yield of transformed cells and with respect to the intracellular localization of the *Arabidopsis* proteins. For practical reasons, in most of the particle bombardment experiments tobacco leaves were transformed. The plastids of epidermal cells in higher plants contain almost undetectable amounts of pigments. Therefore the plastids are not visible in the bright field image (Fig. 3A), but the GFP

fluorescence of the AtStr14 fusion protein indicates their presence and abundance (Fig. 3B). AtStr14 and AtStr16 are soluble proteins, equally distributed in the chloroplasts, similarly to the distribution of the soluble RubisCO protein, whose transit peptide was initially used in GFP control constructs (data not shown).

It has been speculated that Str proteins are involved in iron-sulfur cluster biosynthesis in mitochondria and plastids as a donor of reduced sulfur (Bonomi et al., 1977; Cerletti, 1986). The two-domain AtStr proteins, AtStr1 and AtStr2, are localized in the mitochondrion and the cytoplasm, respectively (Hatzfeld and Saito, 2000; Nakamura et al., 2000; Papenbrock and Schmidt, 2000a, this work). Both proteins belong to the group of 3-mercaptopyruvate AtStrs. Therefore the localization of three AtStr proteins in the plastids provides the basis for new experiments with respect to iron-sulfur cluster biosynthesis. For two out of three plastid-localized single-domain AtStrs, thiosulfate Str activity could already be shown *in vitro* (Bauer and Papenbrock, 2002). Recently, a plastidic NifS-like cysteine desulfhydrase was also suggested to be involved in iron-sulfur cluster biosynthesis (Leon et al., 2002). However, several proteins, both NifS-like and Str, might be involved in providing the reduced sulfur.

The subcellular localization of AtStr15

The AtStr15 protein localization data was unusual: it might be associated with the chloroplast membrane or localized inside the chloroplasts (Fig. 4D). Recently, the intracellular localization of a *N. tabacum* protein, Ntdin, with 56.8% sequence identity to AtStr15 on the amino acid level, was exclusively found in chloroplasts (Yang et al., 2003). The Ntdin protein was evenly distributed in this organelle. The Ntdin and the AtStr15 protein possess high sequence homologies to the Din1 protein from *Raphanus sativus*, the first chloroplast protein detected that was negatively regulated by light. The Din1 protein was shown to be senescence-associated and has sequence similarity to sulfide dehydrogenase or other small stress proteins (Shimada et al., 1998). However, both proteins could be paralogs or orthologs and do not necessarily need to have the same function in the plant cell. Different subcellular localizations of very similar proteins may also occur.

The resolution in cell images visualized by fluorescence microscopy is sufficient for the localization of soluble organellar proteins and can be merged with the autofluorescence

images. However, the investigation of subcellular detail can be drastically improved by the ability to show single nm sections of the cell by CLSM, compared to the fluorescence microscope, in which the fluorescence of the whole protoplast is collected. The highly magnified CLSM images of cells transformed with AtStr15 (Fig. 5E, F) were the grounds for assuming an association with the thylakoid membrane. The images of immunogold labelling by transmission electron microscopy and their statistic evaluation support a preferential association of the AtStr15 molecules to the thylakoid membranes inside the chloroplast. The AtStr15 C-terminus contains a hydrophobic region of about 20 amino acids which is predicted to be transmembrane or membrane-associated (TMHMM, http://www.expasy.ch/tools). Therefore we assume that the protein is attached to the thylakoid membrane by the C-terminus. Studies on a peroxiredoxin protein indicated that the decameric form was attached to the thylakoid membrane, and this depended on the physiological status of the cell (Konig et al., 2002). Future experiments need to be done to explain the function of the membrane-associated AtStr15 protein. The substrate for this putative AtStr might involve another protein which could be activated or deactivated by the transfer of a reduced sulfur.

Computer-based prediction of protein localization is not reliable

The computer-based localization prediction for all AtStrs gave putative localizations in the cytoplasm, chloroplast stroma and thylakoid membrane, mitochondrion, peroxisome and nucleus (Table 2). Our experimental approach revealed that the intracellular computer localization predictions were correct for only three out of six proteins. In a study by Millar et al. (2001), the computer prediction of intracellular localization has been shown to be error prone for individual sequences. Out of a set of 91 proteins detected in purified *Arabidopsis* mitochondria, only 50 to 55 were predicted to be mitochondrial upon analysis with prediction programs. In a high throughput analysis of the *A. thaliana* mitochondrial proteome (Heazlewood et al., 2004), approximately half of the experimental set was predicted to be mitochondrial by targeting prediction programs.

The prediction programs use different algorithms. PSORT is based on an expert system with a knowledge-base and is a collection of 'if-then'-type rules (Nakai and Kanehisa, 1992). TargetP is a neural network-based tool for large-scale intracellular location

prediction of proteins. Using N-terminal sequence information only, it discriminates between proteins destined for the mitochondrion, the chloroplast, the secretory pathway, and "other" localizations with a calculated success rate of 85% (plant) and 90% (non-plant) redundancy-reduced test sets (Emanuelsson et al., 2000). The correctness of the cleavage site prediction was determined to 40-50% for chloroplast transit peptides and mitochondrial pre-sequences, and to above 70% for secretory signal peptides. The algorithms for the localization prediction of plant proteins need to be improved. The occurrence of an additional compartment, the plastid, evolved from an endosymbiotic event and surrounded by a double lipid bilayer, causes many dual-targeting predictions. As more proteins are localized by experimental methods, more targeting peptides will be available as models for the development of new algorithms.

Conclusions

The members of the multi-protein family of *Arabidopsis* Str investigated are localized in the cytoplasm, in the mitochondrion, and in plastids. The mitochondrial localization of AtStr1 was shown by Western blot analysis and transient expression of GFP fusion constructs. The transient transformation of either leaf cells or protoplasts with GFP fusion constructs gave similar results. Three of the single-domain AtStr are translocated to plastids and AtStr15 is closely associated with the thylakoid membrane. The prediction of localization using several computer programs, based on different algorithms, is correct only to about 50%. Therefore biochemical analysis of the intracellular localization of individual proteins is still required in the age of high-throughput methods and bioinformatics.

CHAPTER 7:

GENERAL DISCUSSION

Structure and activity of Strs

Sulfurtransferases/rhodaneses (Strs) have been characterized in many eukaryotic and prokaryotic species investigated to date. Two types of Strs with different substrate specificities for thiosulfate and 3-mercaptopyruvate (3-MP) repectively, have been described until now. Mammalian thiosulfate Strs have been investigated in detail, while the knowledge of 3-MP Strs is still limited. Strs were found as single- and two-domain proteins or in combination with other protein domains (Bordo and Bork, 2002). It was assumed that certain amino acids are essentiell for the specificity of substrate binding, because in mutagenesis studies it was shown that distinct amino acids are indispensable for substrate binding and substrate specificity of 3-MP or thiosulfate, respectively (Nagahara and Nishino, 1996). All Str enzymes contain a highly conserved active site cysteine, which is involved in sulfur transfer reactions (Bordo and Bork, 2002).

The most studied and best characterized two-domain Str is bovine liver thiosulfate Str, which has been the subject of numerous characterizations (Luo et al., 1995; Miller et al., 1991). This enzyme is a single polypeptide of 293 residues and a molecular mass of 33 kDa (Ploegman et al., 1978). The crystal structures of bovine liver thiosulfate Str (Ploegman et al., 1978) and *Azotobacter vinelandii* thiosulfate Str (Bordo et al., 2000) are known. Both enzymes display very similar three-dimensional conformations and are composed of two globular identically folded domains, separated by a connecting loop (Bordo and Bork, 2002). In all described two-domain Strs from eukaryotes the active site cysteine is located in the C-terminal domain, near the interface between the two domains. In the eukaryotic two-domain Str the N-terminal domain was described as an inactive module (Trevino et al., 1998). Very recently a 3-MP Str from *Leishmania major* (Alphey et al., 2003) and SseA, a 3-MP Str from *E. coli* (Spallarossa et al., 2004), were crystallized. Both enzymes display specific structural differences relative to eukaryotic and prokaryotic thiosulfate Strs, concerning the position of the catalytic cysteine residue.

In *Arabidopsis* the Str multi-protein family comprises 18 members including single- and two-domain proteins, which were classified into six groups according to their sequence identities (Bauer and Papenbrock, 2002). Two Strs from group I of this family (AtStr1 and AtStr2) with substrate specificity for 3-MP were isolated and analyzed before this investigation startet (Papenbrock and Schmidt, 2000a, b). AtStr1 consists of 379 amino acids including the targeting sequence and encodes a 35,6 kDa protein, while AtStr2 consists of 366 amino acids including the targeting sequence and encodes a 34,7 kDa protein. Sequences of both mature proteins had an identity of 77,7% (Papenbrock and Schmidt, 2000a, b). Beside many similarities, such as the conserved cysteine residue, the characterized two-domain plant Strs differ in their structure from the two-domain mammalian thiosulfate Strs and 3-MP Strs. The number and positions, and therefore maybe also the function, of cysteine residues vary in plant Str sequences in comparison to known Str sequences from other organisms (Burow et al., 2002). In this work, the search for the physiological role of the two-domain Strs AtStr1 and AtStr2 in the plant organism was continued and the biological characterization of four newly identified single-domain Strs from group VI was started. Single-domain Strs have not been described in any eukaryotic species so far, they were only found in bacteria previously (Ray et al., 2000). Sequences of plant two-domain Strs were described in *Datisca glomerata* (Okubara and Berry, 1999) and in wheat (Niu et al., 2002), but no true thiosulfate Strs have been previously found in plants. By analyzing the four newly identified single-domain Strs, two enzymes with thiosulfate substrate specifity were identified. These two proteins (AtStr16 and AtStr18) were the first single-domain Strs identified in eukaryotic species and further more the first true thiosulfate Strs in *Arabidopsis* described. They are encoded on chromosome 5 in contrast to both two-domain Strs from group I, which are encoded on chromosome 1.

The crystal structure of a single-domain 12 kDa Str from *E. coli* (GlpE) displays radical conformational changes surrounding the active site cysteine, relative to the known three-dimensional structure of two-domain Strs (Spallarossa et al., 2001). Further more in contrast to other two-domain Strs isolated from eukaryotes, the newly identified single-domain thiosulfate Strs from *Arabidopsis* do not contain a second cysteine which was suggested to be involved in acceptor binding close to the active site cysteine (Burow et al., 2002). Obviously, the reaction mechanisms between single- and two-domain Strs differ.

The *in vivo* substrates for sulfur donors of single- and two-domain Strs still remain controversial (Nakamura et al., 2000; Ray et al., 2000). GlpE showed specific Str activity with thiosulfate as substrate, but the affinity to thiosulfate was relatively low (Ray et al., 2000). Further more K_m values for 3-MP Strs estimated in rat revealed that 3-MP is not the naturally occuring substrate (Nagahara et al., 1995). In this work one of the characterized single-domain Strs (AtStr16) revealed a K_m value for thiosulfate in the millimolar range (7,4 mM) which is unphysiologically high, but AtStr18 has a physiologically characteristic K_m value of 1,1 mM. From these results it could be concluded that a search for the physiological substrate of AtStr16 has to be done.

The four closely related proteins from group VI show an average similarity to the C-terminal domain of AtStr1 of about 30% and to the GlpE protein in *E. coli* of about 21%. As mentioned above, the single-domain Strs from *E. coli* are fully active. Even the C-terminal domain of AtStr1 shows specific Str activity alone and is boosted by addition of the N-terminal domain (Burow et al., 2002). To continue the determination of the physiological role of both AtStr1 domains and the interdomain linker sequence in this work, the specific Str activity of the C-terminal domain including the linker sequence was estimated when an increasing amount of modules of the N-terminal domain was added. Surprisingly, there was no higher specific Str activity observed, when the N-terminal domain and the interdomain linker sequence were added in the reaction. Luo and coworkers (1995) demonstrated that the interdomain linker sequence influences the stability and the refolding of the enzyme. Further more single-domain Strs were employed to analyze the structure of Strs in more detail. Structural characterization of a thiosulfate single-domain Str from *E. coli* indicated that the catalytically inactive N-terminal domain found in two-domain thiosulfate and 3-MP Strs is not essential for catalysis (Spallarossa et al., 2001). In this work similar results were observed by analyzing the effect of additional single AtStr1 N-terminal modules on the enzyme activity of the newly identified single-domain Strs from group VI. The purified enzyme AtStr15 showed no Str activity alone and could not be activated by the addition of single AtStr1 N-terminal modules. And also no increasing effect on the enzyme activity of both newly identified thiosulfate single-domain Strs from group VI was found (Bauer and Papenbrock, unpublished). Truncation studies at the N-terminal domain of rhodanese resulted in destabilization of the whole enzyme (Trevino et al., 1998). However, also the C-terminal domain of rhodanese was suggested to be involved in the folding mechanism of the whole protein (Kramer et al., 2001). Due to the tandem

repeat domain architecture it was suggested that two-domain Strs evolved from a common ancestor protein after gene duplication and under the constraint of tertiary structure conservation (Hatzfeld and Saito, 2000). Obviously, both types of Strs found in *Arabidopsis* could have evolved independently in the organism. Overall the physiological role of both domains and the linker remains to be clarified, but it could be interpreted from these results that the interdomain linker sequence has a protective function of the natural substrate binding catalytic pocket. However, the natural substrate has to be identified.

Intracellular localization of Arabidopsis *Strs*

The investigation of the subcellular localization is another tool to elucidate the role of Strs in the organism. Compartmentation plays an important role in regulation and communication of cellular processes, especially in plants (Papenbrock and Grimm, 2001). On the subcellular level, rhodanese from bovine liver was found in the mitochondria (Jarabak and Westley, 1978). In this work 18 Str proteins were identified in *Arabidopsis* as nuclear-encoded proteins. For two of them (AtStr1 and AtStr2) localization studies with different methods had already been done. The AtStr1 protein was shown to be localized in mitochondria, while Western blot analysis and transient expression of GFP fusions indicate that AtStr2 is cytoplasmic (Hatzfeld and Saito, 2000; Nakamura et al., 2000; Papenbrock and Schmidt, 2000a). The use of GFP fusions to investigate the function of targeting sequences offers a rapid, technically simple and non-invasive technique to study targeting *in vivo* (Akashi et al., 1998). For the AtStr1 full-length fusion protein a dual-targeting was observed and also shorter versions of AtStr1 fused to GFP were found to target not only mitochondria, but also chloroplasts. However, on the basis of further localization experiments the authors concluded that the dual-targeting done with different GFP constructs did not reflect the actual subcellular distribution of AtStr1 in the plant cell (Nakamura et al., 2000). Very short fusion constructs might result in artifacts with respect to the intracellular localization (personal communication, Dr. R. Hänsch, Braunschweig). In our hands the fusion of the N-terminal extension of AtStr1 alone to GFP led to non-reproducible results, mainly indicating a cytoplasmic localization (data not shown). To observe the localization in the cell correctly, the full-length proteins including their N-terminal or C-terminal extensions were used to observe the subcellular localization of both proteins from group I and four members of group VI. For the fifth protein identified in group VI (AtStr17)

we assume that it is a pseudogene. As controls the AtStr sequences were ligated into both vectors, pGFP-N and pGFP-C, resulting in fusion proteins with GFP at the C-terminus or the N-terminus of the protein of interest, respectively. However, for all proteins investigated no targeting sequences at the C-terminal domain were observed, as one would expect for peroxisomal or endoplasmatic reticulum-localized proteins (Emanuelsson and von Heijne, 2001). For both proteins from group I our results are in agreement with previous findings, because AtStr1 was shown to be located in mitochondria and AtStr2 in the cytosol using different methods.

For GlpE, the single-domain Str from *E. coli,* it was demonstrated that it is a cytoplasmic protein (Ray et al., 2000). Recently, the intracellular localization of a *N. tabacum* protein, *Ntdin*, with 56.8% sequence identity to AtStr15 on the amino acid level was exclusively found in chloroplasts (Yang et al., 2003). 3 out of 4 single-domain Strs were shown to be chloroplastic. These three enzymes where the first observed Strs in *Arabidopsis*, localized in chloroplasts. In a detailed localization study with GFP fusion proteins from group VI, AtStr14 and AtStr16 were observed as soluble proteins, evenly distributed in the chloroplasts. These results are comparable with the distribution of the soluble RubisCO protein in chloroplasts whose transit peptide was initially used in GFP control constructs. The AtStr15 protein was also localized to the chloroplasts, additional analysis by transmission electron immunmomicroscopy using a monospecific antibody against GFP indicated an attachment to the thylakoid membrane. It was speculated that Str proteins are involved in iron-sulfur cluster biosynthesis in mitochondria and plastids as a donor of reduced sulfur (Bonomi et al., 1977). The knowledge from these studies provides the basis for new experiments with respect to iron-sulfur cluster biosynthesis, because a role of Strs in Fe-S cluster biosynthesis has been discussed for a long time.

Physiological role of Strs in the organism

The physiological roles of Strs are still not resolved and the more Strs with different substrate specificities are being detected in different organisms the stronger are the demands to clarify their function in the organism. The detoxification of cyanide was suggested as the main function of Strs in animals (Westley, 1981) and in plants (Narty, 1970). However, the postulated results did not reveal the clear assurance for an involvement of Strs in this mechanism, at least in plants (Meyer et al., 2003). It was postulated that there is a relationship between the process of cyanide detoxification and

the process of plant senescence (Kasai et al., 1996). Leaf senescence is a genetically controlled and highly regulated process (Qurino et al., 2000). Recently, similarities of the expression patterns of AtStr1 and AtStr2 to senescence-associated proteins were found by sequence comparisons (Papenbrock and Schmidt 2000b). All members from group VI show high sequence similarities to sulfide dehydrogenase (SUD) from *Wolinella succinogenes*, a small stress related protein (Klimmek et al., 1998). A Str from group VI (AtStr15) was described to be a senescence-associated protein (Oh et al., 1996). This protein was shown to be strongly induced by darkness or ethylene treatment. The AtStr15 gene is likely a counterpart of the radish *din1* gene, which also was described to be induced by dark treatment (Oh et al., 1996). Further more AtStr15 shows 56,8% identity to Ntdin, a senescence-associated gene from *N. tabacum* (Yang et al., 2003). Also the AtStr15 protein was highly expressed in aging of *Arabidopsis* plants (Bauer and Papenbrock, unpublished). Because of the sequence homology of the members from group VI among themselves and to the described proteins involved in the senescence process and the observed expression patterns during plant aging, it could be suggested that the single-domain Strs are also associated with plant senescence. In several experiments the process of senescence could be successfully induced by natural aging, by jasmonate methyl ester and darkness in whole plants and detached leaves as demonstrated by the expression of senescence marker genes (Weaver et al., 1998; Miller et al., 1999). In our laboratory different treatment conditions were tested to obtain the expression patterns of *AtStr1* and the specific enzyme activity of Strs under controlled senescence indicating processes in *Arabidopsis*. To compare the expression patterns of *AtStr1* during senescence with typical senescence-associated genes (*SAGs*), two *SAGs* with different expression patterns (*SAG12* and *SAG13*) were also used here. These two genes have been used as senescence marker genes. However, the expression pattern of *AtStr1* differed to that from both typical SAGs analyzed here and might be regulated by a different signal cascade. Still the role of AtStr1 in the senescence process has to be analyzed in more detail. Further on the newly identified Strs from group VI should be investigated in senescence-associated experiments, to observe their role in this context. To elucidate the exact role in this developmental program, a detailed expression analysis during senescence and at different nutritional status (S, N, P) treatments has to be carried out in the future.

To observe an involvement of AtStr1 in cyanide detoxification, plant extracts were treated with raising amounts of cyanide. From the analyzed results the evidence that the

only metabolic role for Strs is a role in cyanide detoxification is very low, because raising the cyanide content by a factor of 5 has neither an effect on the expression of *AtStr1* mRNA and its protein content nor on the specific enzyme activity (Meyer et al., 2003). Similar results were reported from Chew (1973), who observed no correlation between Str activity and the cyanogenic nature of the plant. More recently for PspE (phage-shock protein E), a Str from *E. coli*, it was postulated that cyanide detoxification is not its primary role *in vivo* (Adams et al., 2002). In animals it was described that cyanide detoxification is not the main role of Strs (Long and Brattsen, 1982). In addition the subcellular localization of the putative cyanide detoxification protein AtStr1 and 1-aminocyclopropane-1-carboxylate (ACC) oxidase, which releases cyanide, differ. While ACC oxidase is localized in the cytoplasm (Reinhardt et al., 1994), AtStr1 is localized in the mitochondria (Hatzfeld et al., 2000; Papenbrock and Schmidt, 2000a; this work). It is not clear, whether cyanide is able to diffuse from the cytoplasm to mitochondria, but obtaining the organelle-specific concentrations in *Arabidopsis* might be helpful to clarify the involvement of between 3-MP Strs in cyanide detoxification in more detail. No involvement of single-domain Strs in cyanide detoxification was observed until now, different plant treatment experiments together with the development of mutants and RNAi analysis will help to resolve the physiological role of these enzymes. *Ntdin*, a tobacco senescence-associated gene, was described to be involved in molybdenum cofactor biosynthesis (Yang et al., 2003). Because of the sequence homology of *Ntdin* to the members of group VI it might be helpful to investigate whether these Strs play a role in molybdenum cofactor biosynthesis. Further more, one member of the *Arabidopsis* Strs (AtStr13) was very recently found to be involved in auxin-signalling, which opens a new field of investigation of the functional role by Strs (Zhao et al., 2003).

Conclusions

❖ The biochemical characterization of four single-domain Strs from group VI of the *Arabidopsis* Str multi-protein family allowed the identification of two single-domain Strs with substrate specificity for thiosulfate. These enzymes were the first observed single-domain Strs in any eukaryotic species, and further more the first identified thiosulfate Str in *Arabidopsis*. The investigation of the functional role of

these Strs should be carried on further by the construction of mutants and its analysis through RNAi and metabolic profiling.

❖ Detailed enzyme kinetic studies for both newly identified thiosulfate Strs revealed that neither thiosulfate nor 3-MP are the physiological substrates of these enzymes. An enlarged search for their *in vivo* substrates has to be done in future.

❖ To study intracellular localization of AtStr1 and AtStr2 several methods were applied. For AtStr1, a mitochondrial localization was demonstrated by immunodetection in the proteome of isolated mitochondria resolved by one- and two-dimensional gel electrophoresis and subsequent Western blotting. The respective mature AtStr1 protein was identified by mass spectrometry sequencing. The same result was obtained by transient expression of fusion constructs with GFP in *Arabidopsis* protoplasts, whereas AtStr2 was exclusively localized to the cytoplasm by this method. Three members of the single-domain AtStrs were localized in the chloroplasts as demonstrated by transient expression of GFP fusions in protoplasts and stomata, whereas the single-domain AtStr18 was shown to be cytoplasmic. The remarkable subcellular distribution of AtStr15 was additionally analyzed by transmission electron immunomicroscopy using a monospecific antibody against GFP, indicating an attachment to the thylakoid membrane. These three enzymes where the first observed Strs in higher plants localized in chloroplasts. This knowledge provides the basis for new experiments with respect to the functional analysis.

❖ The physiological role of both domains and the interdomain linker sequence of AtStr1 could not be identified in this work, but it could be interpreted from these results that the interdomain linker sequence has a protective function of the putative natural substrate binding in the catalytic pocket.

❖ It could be concluded that the main physiological role of AtStr1 is not the detoxification of cyanide and a detailed search for the functions of Strs in the organism has to follow.

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Erklärung

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

Hannover, den 19. April 2004   Michael Bauer

Publikationen

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

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

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Mein herzlicher Dank gilt: