Investigation of the role of sulfurtransferases in the metabolism of higher plants

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- Sichtung der Literatur
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- Klonierung und Reinigung der Proteine für die BiFC Untersuchung
- Etablierung und Durchführung der biochemischen Tests
- Reinigung der Proteine für Cross-Linking Experimente
- Erstellung des Manuskripts für die Veröffentlichung

Publikation (Kapitel 4)

Henne M (2013) The role of the sulfurtransferase in the metabolism of higher plants

- Screening der Knock-out Mutanten von *Arabidopsis thaliana*
- Durchführung der Stressversuche und der molekularbiologischen Untersuchung
- Erstellung des Manuskripts für die Veröffentlichung

SUMMARY

Sulfurtransferases (Str) belong to a large family of enzymes, consisting of 20 members in *Arabidopsis thaliana*, which catalyze the transfer of a sulfur atom of suitable sulfur donors to nucleophilic acceptors. Str are found in all organisms of the three domains of life. Although they are widely distributed and a number of functions have been proposed, their biological role is still unknown. Content of this work is the investigation of the function of Str using transgenic plants as well as interaction study of thioredoxin (Trx) with Str.

Using bimolecular fluorescence complementation (BiFC), an interaction of Str and Trx was demonstrated *in vivo*. This interaction seems to be partner and compartment specific. Simply one Str interact with one Trx from the same compartment. AtStr15 was an exception it interacts with four Trx but also from the same compartment.

To confirm the results of the BiFC analysis, enzyme assays were done. One was done on the background of NADPH oxidation and the second was done on the background of H_2S production. But conclusive results could not confirm the interaction biochemically. With started cross-linking experiments the interaction of Trx and Str could be confirmed.

To elucidate the biological function *in vivo* characterization of the *Arabidopsis* T-DNA insertion mutants of AtStr1, AtStr2, AtStr14 and AtStr15 have been started. The investigation of the germination rate on various media showed that thiosulfate and sulfite play an important role in germination.

The conducted expression analyzes could not provide clear evidence that one Str takes over the function of another.

Keywords: BiFC, sulfurtransferase, T-DNA insertion mutants, thioredoxin

ZUSAMMENFASSUNG

Sulfurtransferasen (Str) gehören zu einer großen Enzymfamilie, bestehend aus 20 Mitgliedern in *Arabidopsis thaliana*, die den Transfer eines Schwefelatoms von geeigneten Schwefeldonatoren auf nucleophile Akzeptoren katalysieren. Str kommen in allen Organismen der drei Domänen vor. Obwohl sie weit verbreitet sind und eine Vielzahl an Funktionen vorgeschlagen wurden, ist ihre biologische Rolle weiterhin unbekannt. Inhalt der vorliegenden Arbeit sind die Untersuchungen der Funktion von Str mit Hilfe von transgenen Pflanzen sowie Interaktionsstudie von Str mit Thioredoxinen (Trx).

Mit Hilfe von Bimolekularer Fluoreszenz Komplementierung (BiFC) wurde eine Interaktion von Str und Trx *in vivo* nachgewiesen. Diese Interaktion ist offenbar spezifisch für bestimmte Interaktionspaare und die jeweiligen Kompartimente. Nur genau eine Str interagiert mit einem Trx aus demselben Kompartiment. AtStr15 ist eine Ausnahme, es interagiert mit vier Trx, aber auch aus dem gleichen Kompartiment.

Um die BiFC Analysen zu bestätigen, wurden Enzymtests durchgeführt. Ein Test wurde auf den Hintergrund der NADPH Oxidation und ein Test wurde auf dem Hintergrund der H_2S Produktion durchgeführt. Doch eindeutige Ergebnisse konnten diese Tests nicht liefern. Mit Cross-linking Experimente, die begonnen wurden, konnte die Interaktion von Str und Trx nachgewiesen werden.

Um die biologische Funktion *in vivo* zu klären, wurde die Charakterisierung von *Arabidopsis* T-DNA Insertionsmutanten von AtStr1, AtStr2, AtStr14 und AtStr15 begonnen. Die Untersuchung der Keimungsrate auf verschiedenen Medien hat gezeigt, dass Thiosulfat und Sulfit eine wichtige Rolle während der Keimung spielen.

Die durchgeführten Expressionsanalysen konnten keine klaren Beweise dafür liefern, dass eine Str die Funktion einer anderen übernimmt, wie z. B. AtStr2 anstelle von AtStr1 aktiv ist und umgekehrt, sollte diese ausgeschaltet sein.

Schlüsselwörter: BiFC, Sulfurtransferasen, T-DNA Insertionsmutanten, Thioredoxin.

ABBREVIATIONS

3-MP	3-mercaptopyruvate
3-MST	3-mercaptopyruvate sulfurtransferase
aa	amino acids
Acc. no.	accession number
AR	arsenate reductase
AtStr	*Arabidopsis thaliana* sulfurtransferase
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BiFC	bimolecular fluorescence complementation
BS3	bissulfosuccinimidyl suberate
BSA	bovine serum albumin
CAS	β-cyano-L-alanine synthase
CE	capillary electrophoresis
CLSM	confocal laser scanning microscope
CP	chloroplast
Cys	cysteine
cyt	cytosol
db	database
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphate
DTE	dithioerythritol
DTNB	5,5-dithiobis-2-nitrobenzoic acid
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetraacetic acid
FW	fresh weight
GSH	glutathione reduced
H_2S	hydrogen sulfide
IPTG	isopropyl-1-thio-β-D-galactoside
KCN	potassium cyanide
kDa	kilo Dalton
LB	Luria-Bertani (medium)
Moco	molybdenum cofactor
MPF	multi-protein family

MS	Murashige & Skoog (medium)
NADPH	nicotinamide adenine dinucleotide phosphate
NBT	nitroblue tetrazolium
OD	optical density
PCR	polymerase chain reaction
PEG	polyethylene glycol
Rhd	rhodanese
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
Str	sulfurtransferase(s)
TCA	trichloroacetic acis
TCEP	tris-(2-carboxyethyl)-phosphine
T-DNA	transfer deoxyribonucleic acid
TP	target peptide
Tris	2-amino-2-hydroxymethyl-propane-1,3-diol
Trx	thioredoxin(s)
Trx-Red / Red	thioredoxin reductase
TS	thiosulfate
wtcol	wild-type ecotype Columbia
YFP	yellow fluorescent protein

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CHAPTER 1

GENERAL INTRODUCTION

Sulfurtransferases

Sulfurtransferases (Str; EC 2.8.1.x) belong to a group of enzymes widely distributed in archaea, eubacteria and eukaryotes, that catalyze the transfer of a sulfur atom from suitable sulfur donors to nucleophilic sulfur acceptors. The most studied and best characterized Str is the bovine liver rhodanese (Rhd) (thiosulfate: cyanide Str, EC 2.8.1.1) which catalyze, *in vitro*, the transfer of a sulfane sulfur atom from thiosulfate (TS) to cyanide, leading to the formation of sulfite and thiocyanate (Westley, 1973)

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

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

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

According to the generally accepted mechanism, during catalysis the enzyme cycles between two distinct forms, the free enzyme (Rhod) and a covalent enzyme-sulfur intermediate (Rhod-S) (Fig. 1). The original crystal structure of bovine liver rhodanese (Ploegman et al., 1978) and other crystallographic investigations (Gliubich et al., 1996) have shown that the Rhod-S intermediate is characterized by a persulfide bond at the sulfhydryl-group of the essential residue cysteine (Cys) 247. A number of molecules can serve as sulfur donors in the Str reaction, such as TS, thiosulfonate, persulfides and 3-mercaptopyruvate (3-MP). Several compounds, such as cyanide, thiols and dithiols can act as sulfur acceptors (see Fig. 3 in Chapter 2). The determination of the *in vitro* activity was done with 3-MP or TS as donor and cyanide as acceptor. However, the natural substrates acting as sulfur donor and acceptor have not been identified so far and their identification would help to understand the physiological function of Str.

In *Arabidopsis thaliana* 20 putative members of this enzyme family exist and are located in different compartments (Bauer et al., 2004; Peltier et al., 2004). Based on their sequence homology they are divided into six groups and have at least one rhodanese-domain. Although they are widely distributed, the physiological role of Str is unknown yet. Different functions have been suggested for them. While in mammals rhodanese may be involved in the

elimination of toxic cyanogenic compounds to the nontoxic thiocyanate (Vennesland et al., 1982; Nagahara et al., 1999), its ubiquity suggests additional physiological functions (Chew, 1973). AtStr1 and AtStr2 may be involved in seed and embryo development (Mao et al., 2011). AtStr5 has been identified as Cdc25, a small dual specificity tyrosine phosphatase that is involved in the dephosphorylation of the cyclin-CDK complexes for the progression of the cellcycle (Landrieu et al., 2004a; 2004b). It has been suggested that Str play a role in arsenate reduction (Bleeker et al., 2006; Dhankher et al., 2006). 3-mercaptopyruvate sulfurtransferase (3-MST) catalyzes a transsulfuration from 3-MP to pyruvate in the degradation process of cysteine. On the one hand, Stoichiometric concentration of hydrogen peroxide or tetrathionate attacked the catalytic Cys^{247}, to inhibit rat MST and on the other hand dithithreitol or thioredoxin (Trx) restored the activity (Nagahara et al., 1995; Nagahara and Nishino, 1996). Recent studies showed that Str are involved in sulfite networks to produce TS in the presence of sulfite and 3-MP (Brychkova et al., 2013).

Interaction of sulfurtransferase with other proteins

A homologue of AtStr15 in tobacco N receptor-interacting protein 1 (NRIP1) interacts with both N's TIR domain and p50 of the *Tobacco mosaic virus*. Interestingly, NRIP1 that normally localizes to the chloroplasts is recruited to the cytoplasm and nucleus by the p50 effector and an immune response follows. As a consequence, NRIP1 interacts with N only in the presence of the p50 effector (Caplan et al., 2008). Another working group showed that the sulfur-binding donating protein (SdbP), a rhodanese homologue from *Aquifex aeolicus,* interacts with a sulfur reductase (SR) and sulfur oxygenase reductase (SOR) (Aussignargues et al., 2012). IscC (iron-sulfur cluster) is one of the three *Escherichia coli* (*E. coli*) L-cysteine desulfurases. It interacts with FdhD which was discovered to be a Str and which is essential for the activity of formate dehydrogenase (Thomé et al., 2012). The interaction of IscC with FdhD results in a sulfur transfer between IscC and FdhD in the form of persulfides (Thomé et al., 2012).

Thioredoxins

Trx are small (~12-14 kDa), widely distributed proteins that function as protein disulfide oxidoreductases through the reversible oxidation of two Cys thiols in a structurally conserved active site (WCXXC) (Holmgren, 1985). Trx are able to reduce disulfide bridges between two Cys residues present in peptides by using a so-called dithiol mechanism. The reduction process always begins with the first Cys of the redox site, which is called the catalytic Cys,

releasing a thiol on the target protein and forming a disulfide bond with the second target Cys. In a second step, the second Cys (resolving Cys) of the Trx reduces the intermediate complex, releasing the reduced target and the oxidized Trx (Meyer et al., 2009; 2012). In its reduced form, Trx can function as a hydrogen donor or as a regulatory factor for various target proteins like metabolic enzymes, redox proteins, transcription factors or MAP kinases (Arnér and Holmgren, 2000). Two types of Trx systems have been described in plants based on the source of reducing power: the ferredoxin/Trx reductase system located in the chloroplasts and the extraplastidic NADP/Trx reductase (Schürmann and Jacquot, 2000). There are more than 40 Trx or Trx-like genes in *Arabidopsis thaliana* (Meyer et al., 2005). Trx are classified based on primary structures and subcellular localization into six groups. Trx f, m, x and y are found in chloroplast, whereas Trx o and h are localized to the mitochondrion or cytoplasm (Gelhaye et al., 2004; Meyer et al., 2005). Although all Trx are of comparable size and appear to have very similar redox properties, they fulfill specific functions. This specificity seems to be based on structural complementarity, which allows specific interaction between the different Trx and their respective target proteins (Schürmann and Jacquot, 2000).

Interaction of sulfurtransferase with thioredoxin

It has been suggested that Str have a role in the maintenance of redox homeostasis by virtue of its ability to interact with Trx. It is now an established fact that Str utilize reduced Trx efficiently as sulfur acceptor substrates (Nandi and Westley, 1998). The *glpE* gene of *E. coli* encodes Str and its kinetic analysis has revealed that its mechanism of catalysis is a double-displacement mechanism that takes place by way of an enzyme-sulfur intermediate and requires an active-site Cys. It has moreover suggested that Trx could be physiological substrates for Str (Ray et al., 2000). Cytosolic 3-MST of *Leishmania major* and *Leishmania mexicana* can also oxidize reduced Trx by using it as the accepting nucleophile (Williams et al., 2003). Oxidative stress decreases 3-MST activity so as to increase the amount of Cys, a precursor of Trx or glutathione, and furthermore, these cellular reductants restore the activity (Nagahara and Katayama, 2005), thereby suggesting that rat Str are involved in the maintenance of redox homeostasis by interacting with Trx. Reduced Trx turn on a redox switch for the enzymatic activation of rat 3-MST which contributes to the maintenance of cellular redox homeostasis in rat cells (Nagahara et al., 2007). *In vitro* experiments using fluorescence spectroscopy, kinetic studies and mass spectrometry analysis showed that sodium 2-propenyl thiosulfate, which induces apoptosis in cancer cells, inhibits Str activity in tumor cells by thiolation of the catalytic Cys. Trx restores the enzyme activity in a

concentration-dependent and time-dependent manner (Sabelli et al., 2008). A study using a proteomics approach has identified 50 potential Trx target proteins of plant mitochondria and 3-MST is among these target proteins of Trx (Balmer et al., 2004). In *Trichomonas vaginalis* Trx release H_2S from persulfide provided by 3-MP at the active site of 3-MST (Mikami et al., 2011).

Protein-protein interaction has been analyzed by bimolecular fluorescence complementation (BiFC) (Walter et al., 2004; Bhat et al., 2006). The BiFC assay is based on the observation that N- and C-terminal of YFP do not reconstitute a functional fluorophore. However, if fused with two interacting proteins, the two non-functional halves of the fluorophores are brought into tight contact, refold together and generate *de novo* fluorescence (Bhat et al., 2006). As described in Walter et al. (2004) the vector pUC-SPYNE and pUC-SPYCE (abbreviation for split YFP N-terminal/C-terminal fragment expression) were used for the protein-protein interaction of Trx and Str *in planta*.

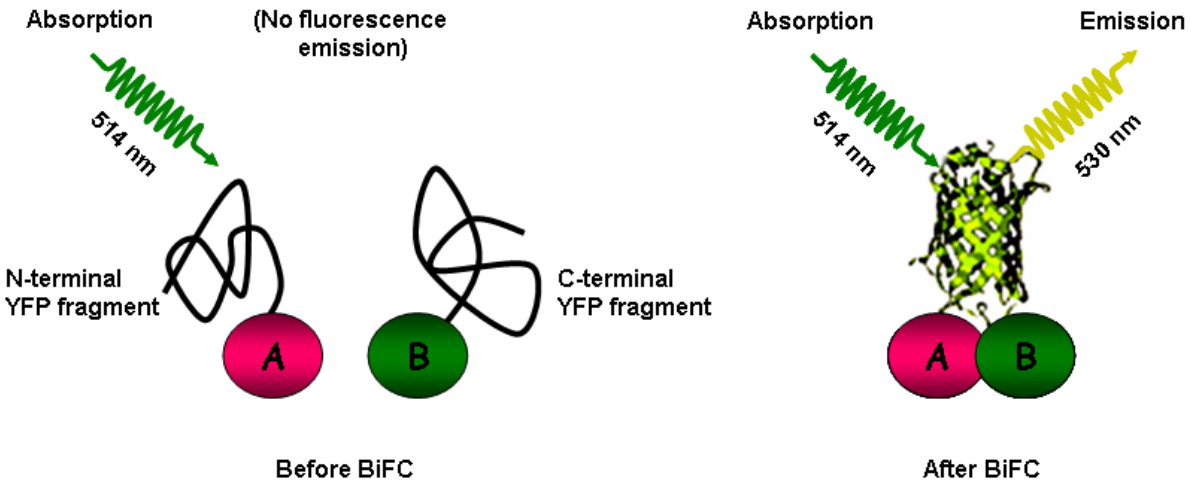


Figure 2. Principle of the BiFC assay. The scheme depicts the principle of the BiFC assay, exemplified by a split YFP fluorophore. Proteins A and B are fused to N- and C-terminal fragments of YFP, respectively. In the absence of an interaction between A and B, the fluorophore halves remain non-functional. Following interaction between A and B, a functional fluorophore is reconstituted which exhibits emission of fluorescence upon excitation with an appropriate wavelength (Bhat et al., 2006).

Aim of this thesis

- Investigations on the interaction of Str and Trx by using bimolecular fluorescence complementation (BiFC)
- Heterologous expression in *E. coli* of five different Str and four different Trx, purifications of the proteins and analyzing the specificity of their interaction with biochemical assays.
- Characterization of *Arabidopsis* T-DNA insertion mutants and investigations on their behavior under different stress situations.
- Investigations of the mutants with different molecular biological methods
- Functional analysis of *atstr2* and *atstr14* by expression analysis under different stress conditions.

CHAPTER 2

LATEST NEWS ABOUT THE SULFURTRANSFERASE PROTEIN FAMILY OF HIGHER PLANTS

PAPENBROCK J, GURETZKI S, HENNE M (2010)

AMINO ACIDS **41**: 43–57

Abstract

Sulfurtransferases/rhodaneses (Str) comprise a group of enzymes widely distributed in all phyla which catalyze *in vitro* the transfer of a sulfur atom from suitable sulfur donors to nucleophilic sulfur acceptors. The 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. Plants as well as other organisms contain many proteins carrying a typical rhodanese pattern or domain forming multi-protein families (MPF). Despite the presence of Str activities in many living organisms, the physiological role of the members of this MPF has not been established unambiguously. While in mammals these proteins are involved in the elimination of toxic cyanogenic compounds, their ubiquity suggests additional physiological functions. In plants, Str are localized in the cytoplasm, in mitochondria, in plastids and in the nucleus. Str probably also transfer reduced sulfur onto substrates as large as peptides or proteins. Several studies in different organisms demonstrate a protein-protein interaction with members of the thioredoxin MPF indicating a role of Str in maintenance of the cellular redox homeostasis. The increased expression of several members of the Str MPF in various stress conditions could be a response to oxidative stress. In summary, data indicate that Str are involved in various essential metabolic reactions.

INTRODUCTION

Sulfurtransferase/rhodanese (Str) enzymes catalyze the transfer of a sulfur atom from suitable sulfur donors to nucleophilic sulfur acceptors. The most studied and best characterized Str is bovine rhodanese which catalyzes *in vitro* the transfer of a sulfane sulfur atom from thiosulfate (TS) to cyanide, leading to the formation of sulfite and thiocyanate. Rhodanese, from the German word for thiocyanate, "Rhodanid", is a widespread enzyme. Rhodanese activity has been detected in all major phyla (http://www.ncbi.nlm.nih.gov/cgi-bin/COG, COG0607). Bovine liver rhodanese has been the object of numerous functional investigations (Westley, 1973; 1981). According to the generally accepted mechanism, during catalysis the enzyme cycles between two distinct forms, the free enzyme (Rhod), and a covalent enzyme-sulfur intermediate (Rhod-S):

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

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

The original crystal structure of bovine liver rhodanese (Ploegman et al., 1978; Russel et al., 1978) and other independent crystallographic investigations (Gliubich et al., 1996) have shown that the Rhod-S intermediate is characterized by a persulfide bond at the sulfhydryl group of the essential cysteine (Cys) residue 247. The shape and properties of the proteins surface in the proximity of the active site are considered essential for substrate binding (Luo and Horowitz, 1994). In this respect the alignment of the known amino acid sequences of rhodanese enzymes indicates a high overall sequence homology. The tertiary structure of bovine rhodanese is composed of two domains which are characterized by very similar three-dimensional folds in spite of a negligible overall sequence homology. The structural similarity of the two rhodanese domains has been considered as the prototype of divergent evolution from a common ancestor protein which, after gene duplication and under the constraint of tertiary structure conservation, led to the almost complete obliteration of sequence similarity between the N- and the C-terminal halves (Ploegman et al., 1978; Bordo et al., 2000).

It was shown that rhodanese domains are structural modules found as one-domain proteins, as tandemly repeated modules in which the C-terminal domain only bears the properly structured active site, or as members of multi domain proteins. More than 8,000 sequences containing a rhodanese domain (PF00581) have been classified so far. In *Escherichia coli* (*E. coli*) several genes encoding proteins consisting of (or containing) a rhodanese domain bearing the potentially catalytic Cys have been identified. After the crystal structure of one of these

proteins, the 12-kDa GlpE protein, has been solved, it can be considered to be the prototype structure for the ubiquitous one-domain rhodanese module (Ray et al., 2000; Spallarossa et al., 2001).

In many prokaryotes and eukaryotes 3-mercaptopyruvate (3-MP) Str activity was discovered. Both Str proteins, 3-MP Str and rhodanese, isolated from the same organism (here: rat) accepted 3-MP and TS but the ratios of their respective enzyme activities differed. The purified as well as the recombinant rat 3-MP Str revealed K_m values for 3-MP in the low millimolar range. This review focuses on enzymes in the categories EC 2.8.1.1, EC 2.8.1.2, and EC 2.8.1.3 (http://us.expasy.org/enzyme/).

General description of plant sulfurtransferases

Members of this protein family were described quite early in the biochemical history. Already in 1938 rhodanese activity was found to be present in plants (Gemeinhardt, 1938), however, closer investigations were undertaken much later. Str activity was compared in crude extracts of cyanogenic and non-cyanogenic plants (Chew, 1973; Kakes and Hakvoort, 1992). Diurnal variation of cyanogenic glucosides, thiocyanate and rhodanese activity was measured in the cyanogenic plant *Manihot esculenta* (Okolie and Obasi, 1993). In 1984 Schmidt and coworkers started first attempts to purify 3-MP Str (Schmidt, 1984) and TS Str (Schmidt et al., 1984) from photosynthetic organisms. Three different fractions containing Str activity were analyzed from the green alga *Chlorella fusca* (Schmidt et al., 1984). In *Chlamydomonas reinhardtii* TS reductase and rhodanese activities were postulated to be catalyzed by one enzyme (Prieto et al., 1997).

Sequencing of the complete *Arabidopsis* genome accelerated the analysis of plant Str on the molecular level. Independently, three groups isolated and analyzed two Str from *Arabidopsis* and called them either TS Str (Hatzfeld and Saito, 2000) or 3-MP Str (Nakamura et al., 2000; Papenbrock and Schmidt, 2000a; 2000b). Both sequences coding for Str1 and Str2 from *Arabidopsis* (AtStr1, AtStr2) evolved probably by gene duplication. Database mining revealed the existence of 20 different Str or Str-like proteins of different length in *Arabidopsis* (Bauer and Papenbrock, 2002; Bartels et al., 2007; Table 1).

Table 1. Overview of the 20 members of the sulfurtransferase/rhodanese multi-protein family in *Arabidopsis thaliana*. The protein name, gene identification, number of amino acids, predicted or experimentally shown localisation and remarks on different aspects are summarised including the respective references. The programs Predotar, PSORT, and TargetP were used for the localisation prediction (http://www.expasy.ch/tools). Abbreviations: aa, amino acids; CP, chloroplast; db, database; ER, endoplasmatic reticulum; exp, experimental; ID, identification; MT, mitochondrial; pred, predicted.

	AGI ID	Amino acids	Localisation (pred/exp)	Reference for localization	Putative *in vivo* or *in vitro* enzyme activity	Reference
Group I						
1	At1g79230	322	MT (exp)	Bauer et al., 2004 Heazlewood et al., 2004 Nakamura et al., 2000	TS Str 3-MP Str	Papenbrock and Schmidt, 2000a, 2000b Hatzfeld and Saito, 2000 Nakamura et al., 2000
2	At1g16460	318	Cyt (exp)	Bauer et al., 2004 Hatzfeld and Saito, 2000 Nakamura et al., 2000	TS Str 3-MP Str	
Group II						
3	At5g23060	387	MT (exp)	Heazlewood et al., 2004	similar to unknown protein	db annotation
4	At4g01050	457	CP, thylakoid membrane (exp)	Peltier et al., 2004	hydroxyproline-rich glycoprotein	db annotation
4a	At3g25480	264	CP, thylakoid membrane (exp)	Peltier et al., 2004	hypothetical protein	db annotation
Group III						
5	At5g03455	132	nucleus (pred/exp)	Landrieu et al., 2004	dual-specificity tyrosine phosphatase Acr2 AR	Landrieu et al., 2004 Duan et al., 2005
6	At1g09280	581	Cyt (pred/exp)	www.expasy.ch	Unknown protein	db annotation
7	At2g40760	522	MT (pred)	www.expasy.ch	unknown protein	db annotation
8	At1g17850	366	CP/ER (pred)	www.expasy.ch	contains rhodanese-like PF|00581 domain	db annotation
Group IV						
9	At2g42220	234	CP, thylakoid membrane (exp)	Peltier et al., 2004 Bartels, 2006	*Datura innoxia* homolog Cd^{2+} induced	Louie et al., 2003
10	At3g08920	214	MT (pred/preliminary exp)	Bartels, 2006	unknown protein	db annotation
11	At4g24750	260	CP/Per (pred/preliminary exp)	Bartels, 2006	putative protein	db annotation
Group V						
12	At5g19370	309	CP/MT (pred)	www.expasy.ch	putative peptidyl-prolyl cis-trans isomerase	db annotation Zhao et al., 2003
13	At5g55130	464	CP/Cyt (pred)	www.expasy.ch	molybdopterin synthase sulfurylase	db annotation
Group VI						
14	At4g27700	237	CP (exp)	Bauer et al., 2004 Peltier et al., 2004	hypothetical protein	db annotation
15	At4g35770	182	CP, thylakoid membrane (exp)	Bauer et al., 2004	AtSen1, senescence association, dark-induced, MoCo synthesis TS Str	Oh et al., 1996 Schenk et al., 2005 Papenbrock, unbublished
16	At5g66040	120	CP (exp)	Bauer et al., 2004	senescence-associated protein sen1-like protein; ketoconazole resistance protein-like TS Str	db annotation Bauer and Papenbrock, 2002
17	At2g17850	150	Nu (pred)	www.expasy.ch	putative senescence-associated rhodanese protein; similarity to Ntdin homology to defense and stress associated *Cucurbita* proteins	Yang et al., 2003 Walz et al., 2004
17a	At2g21045	169	MT/Cyt (pred)	www.expasy.ch	senescence-associated protein	db annotation
18	At5g66170	136	Cyt (exp)	Bauer et al., 2004	senescence-associated protein sen1-like protein TS Str	db annotation Bauer and Papenbrock, 2002

Next to *Arabidopsis*, the first sequence from plant Str DNA sequences was published from *Datisca glomerata*, a Datiscaceae living in symbiosis with nitrogen-fixing bacteria (Okubara and Berry, 1999). Then a full length Str sequence from wheat was annotated in the database (Accession number (Acc. no.) AAK64575) which shows 62 % identity and 78 % similarity to AtStr1. Meanwhile, members of the rhodanese family were identified in various plants by the use of databases (e.g. http://smart.embl-heidelberg.de/). In comparison to *Arabidopsis* (AtStr1) the *Brassica napus* Str shows an identity of 75.1% and a similarity of 81.4% in an alignment (http://www.ebi.ac.uk/Tools/emboss/align/), *Brassica oleracea* 76.9%/86.5%, *Arabidopsis* (AtStr.2) 68.6%/76%, *Populus trichocarpa* 70.8%/81.5%, *Vitis vinifera* 71.4%/82.8%, *Dactisca glomerata* 69.5%/79.7%, *Oryza sativa* 61.1%/72.2%, *Zea mays* 50.0%/63.5%, *Physcomitrella patens* 42.1%/55%, *Ostreococcus lucimarinus* 34%/47.5%, *Ostreococcus tauri* 35.4%/49.1%, *Chlamydomonas reinhardtii* 32.6%/49.2% and *Picea sitchensis* 53.9%/68.4%. A phylogram (http://www.ebi.ac.uk/Tools/clustalw2/) estimated with these sequences shows three main groups (Fig. 1). Group 1 consists of two subgroups, namely Liliopsida (1a) and eudicotyledons (1b), group 2 comprises green algae and the moss *Physcomitrella patens*, and group 3 consists of only *Picea sitchensis*, belonging to the Coniferophyta reflecting the taxonomy and the average number of sequences in each taxon.

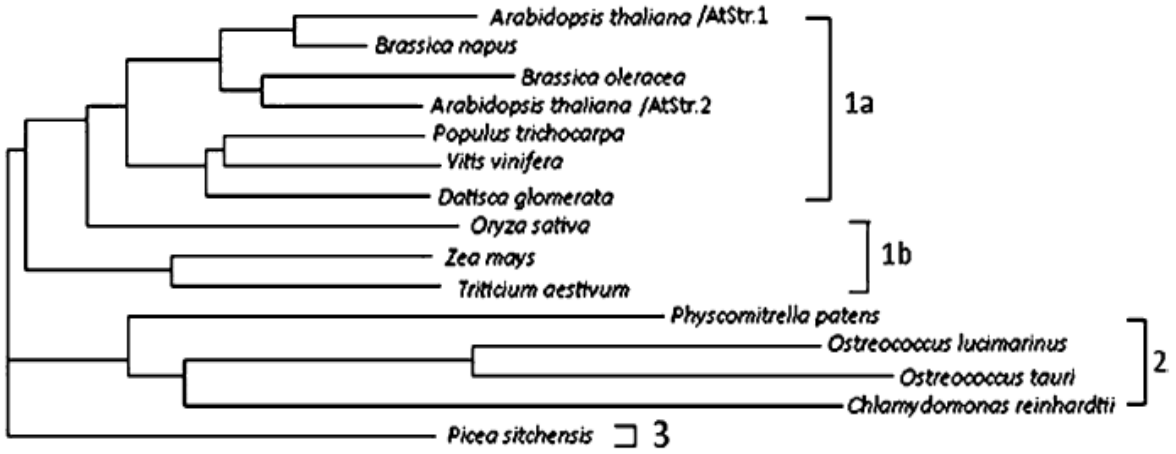


Figure 1. The phylogram was obtained using the following protein sequences of two-domain Str in the Clustalw2 program (http://www.ebi.ac.uk/Tools/clustalw2/, Method: Neighbour-joining): *Arabidopsis thaliana* (L.) Heynh. (AtStr1: O64530; AtStr2: Q9S7Y9), *Brassica napus* L. (Papenbrock unpublished), *Brassica oleracea* L. (B2D2I1), *Populus trichocarpa* Torr. & A. Gray (A9PCY9), *Vitis vinifera* L. (A5B8K7), *Dactisca glomerata* K. Presl Baill. (Q9ZPK0), *Oryza sativa* (LOC_Os12g41500, http://rice.plantbiology.msu.edu/), *Zea mays* L. (B4FPY3)**,** *Triticium aestivum* L. (Q94C43), *Physcomitrella patens* (Hedw.) Bruch & Schimp. (A9SGZ1), *Ostreococcus lucimarinus* strain CCE9901 (A4S6Y9), *Ostreococcus tauri* C. Courties & M.-J. Chrétiennot-Dinet (Q00VJ9), *Chlamydomonas reinhardtii* P.A. Dangeard **(**A8JB06), and *Picea sitchensis* (Bong.) Carr. (9NWI8).

The multi-protein Str family in Arabidopsis

Since in December 2000 the complete sequence of *Arabidopsis* was published (The Arabidopsis Genome Initiative, 2000) a comprehensive characterization of the multi-protein family (MPF) could be started. In addition to the 18 Str identified previously (Bauer and Papenbrock, 2002), recent data mining of *Arabidopsis* databases revealed the appearance of two more sequences containing one typical rhodanese domain. Therefore, the phylogenetic tree of AtStr had to be expanded by two members, AtStr4a and AtStr17a. All AtStr proteins have been classified in six groups according to their amino acid sequence homologies (Bartels et al., 2007; Table 1). Rhodanese domains are visualized within the Str protein sequences (Acc. No. SM00450) (http://smart.embl-heidelberg.de/). In Figure 2 schematic diagrams of representative Str proteins occurring in *Arabidopsis* and containing rhodanese domain(s) are shown.

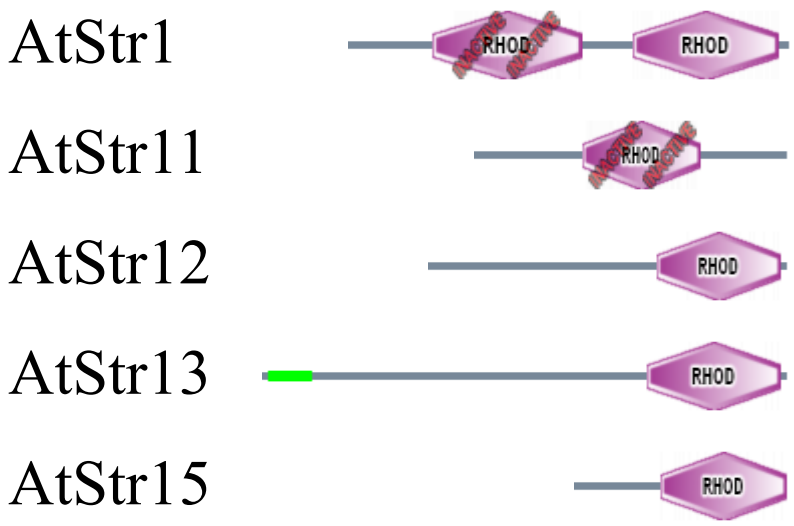


Figure 2. Schematic diagrams of representative Str proteins containing rhodanese domain(s) occurring in *Arabidopsis*. The accession number for the rhodanese domain in SMART is SM00450 (http://smart.embl-heidelberg.de/).

Localization of Str in Arabidopsis cells

The knowledge about the localization of a protein in the organism and in the cell often helps to elucidate its function. Compartmentalization plays an important role in regulation and

communication of cellular processes, especially in plants (Papenbrock and Grimm, 2001). In contrast to cells of heterotrophic eukaryotes, plant cells contain, besides mitochondria, one additional type of semi-autonomous organelles enveloped by two membranes, the plastids. The bacterial Str analyzed so far are localized either in the periplasm or in the cytoplasm. The GlpE protein from *E. coli* is a cytoplasmic protein (Ray et al., 2000). A rhodanese-like protein from *Synechococcus* sp. strain PCC 7942 is localized in the periplasm (Laudenbach et al., 1991). In mammalian cells 3-MP Str was found in both mitochondria and cytoplasm whereas the subcellular localization of rhodanese proteins was exclusively in the mitochondria (Westley, 1973; Jarabak and Westley, 1978; Nagahara et al., 1998). A targeting sequence for the rat rhodanese was elucidated which forms an amphipathic α-helix. However, after the protein has been transported into the mitochondrial matrix space the targeting sequence is not cleaved (Waltner and Weiner, 1995). Rat 3-MP Str contains also a putative targeting signal; the retention in the cytoplasm might be controlled by posttranslational modification such as phosphorylation/dephosphorylation (Nagahara et al., 1999).

Different regions of the N-terminal parts of AtStr1 and AtStr2 were fused to the green fluorescent protein (GFP) (Nakamura et al., 2000). The results demonstrated a subcellular localization for AtStr1 and AtStr2 in the cytoplasm and in mitochondria, in agreement with immunoblots done in parallel. Some regions of AtStr1 fused to the green fluorescent protein were found to target not only in mitochondria, but also in the chloroplasts, suggesting that the regions of the targeting sequence recognized by protein import systems of mitochondria and chloroplasts are not identical (Nakamura et al., 2000). Other groups using the same methodology demonstrated the localization of AtStr1 in mitochondria and of AtStr2 in the cytoplasm (Hatzfeld and Saito, 2000; Bauer et al., 2004).

AtStr14 and AtStr16 are localized in the chloroplasts whereas AtStr18 remains in the cytoplasm (Bauer et al., 2004). The localization of AtStr15 protein is unusual. It was not clear if the protein was outside the chloroplast, on the chloroplast envelope or attached to the thylakoid membrane. A higher magnification indicated an association of AtStr15 with the thylakoid membrane confirmed by immune gold labeling approaches (Bauer et al., 2004). In a modified three-phase partitioning approach to isolate thylakoid membrane proteins, 4 out of 242, contained a rhodanese domain (At4g01050, AtStr4; At3g25480, AtStr4a with one membrane spanning region; At2g42220, AtStr9; At4g27700, AtStr14) (Peltier et al., 2004). These results are in agreement with predictions by programs and with experimental results

obtained with GFP fusions (Bauer et al., 2004; Table 1). In summary, for 14 out of 20 AtStr the localization was experimentally determined (for some proteins still preliminary) by different means (e.g. Bauer et al., 2004).

Pattern and profiles in Arabidopsis Str

The most important and unifying amino acid in all members of the Str MPF is a Cys residue in the C-terminal domain surrounded by a certain environment to form the active site. In addition all proteins in the Str family are unified by well-defined highly conserved sequence domains. Because of the ubiquitous distribution of Str in eubacteria, archaea, and eukaryotes a large number of Str sequences are available. The search algorithms for typical conserved sequence domains were defined already some time ago and could be proven and improved in many approaches. In PROSITE two patterns were developed for the rhodanese family: consensus pattern 1 (FY)-x(3)-H-(LIV)-P-G-A-x(2)-(LIVF) (Acc. No. PS00380) and consensus pattern 2 (AV)-x(2)-(FY)-(DEAP)-G-(GSA)-(WF)-x-E-(FYW) (Acc. No. PS00683). They are based on highly conserved regions, one is located in the N-terminal region, the other at the C-terminal extremity of the protein (http://www.expasy.ch). Only the rhodanese C-terminal pattern can be identified in all Str-like proteins.
In InterPro two entries were developed (http://www.ebi.ac.uk/interpro/): IPR001307 recognizes only TS Str (rhodanese-like, matches 1475 proteins, 12th November 2009) whereas in the pattern defined in IPR001763 (Rhodanese/Cdc25 fold, matches 11334 proteins, 12th November 2009) two rhodanese domains can be identified. This entry comprises similarities to the catalytic domain of Cdc25 phosphatase, the non-catalytic domains of eukaryotic dual-specificity MAP-kinase phosphatases, the non-catalytic domains of yeast PTP-type MAP-kinase phosphatases, the non-catalytic domains of yeast Ubp4, Ubp5, Ubp7, the non-catalytic domains of mammalian Ubp-Y, the *Drosophila* heat shock protein HSP-67BB, several bacterial cold-shock and phage-shock proteins, plant senescence-associated proteins, catalytic and non-catalytic domains of rhodanese.

In two-domain Str the N- and C-terminal domain are connected by a linker. In plant Str the linker sequence is exceptionally longer than in sequences from other species (Burow et al., 2002). The one-domain Str from bacteria are fully active and show high similarity to the C-terminal domain of two-domain Str. These facts raise the question whether each domain of

the two-domain AtStr might form an active Str enzyme by itself: Enzymatic activity of the AtStr1 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). In *Arabidopsis* a number of proteins with one rhodanese domain were identified (Bauer and Papenbrock, 2002; Bartels et al., 2007b). At least three of the small one-domain Str-like proteins similar to the one-domain Str from bacteria showed high TS Str activity (Bauer and Papenbrock, 2002; Bartels, 2006).

Besides many similarities, such as the conserved Cys residue, the characterised two-domain plant Str differ in their structure from the two-domain mammalian TS and 3-MP Str. The number and positions of Cys residues, and consequently maybe also the function in plant Str, are different from known Str sequences in other organisms (Burow et al., 2002). In spectroscopic analyses of recombinant AtStr1C332S it could be shown that the reported loss of Str activity in this mutant (Burow et al., 2002) is due not only to the loss of the persulfuration site but also due to conformational changes of the whole protein structure (Bartels et al., 2007a). Of the five Cys residues in AtStr1 a second Cys (C339) close to the catalytic C332 was suggested to be involved in catalysis. The Str activity of the AtStr1C339V mutant was shown to be reduced to 25% using TS as sulfur donor substrate and slightly but significantly using 3-MP (Burow et al., 2002). The substitution of C339 by valine did not alter the conformation of the protein as shown by spectroscopic analyses (Bartels et al., 2007a), thus supporting a catalytic rather than a structural role of C339. The Cys residue might act in recognising and binding of the acceptor molecule in close vicinity to the active site. Determination of the three-dimensional structure of the AtStr1C339V mutant might clarify the role of C339 unambiguously.

The activity of an AtStr1 derivative with a shortened linker sequence was reduced by more than 60% in comparison to the wild-type activity, probably because of a drastically reduced protein stability (Burow et al., 2002). The mutant was demonstrated to be far more prone to proteolytic digestion by trypsin than the wild-type AtStr1 (Bartels et al., 2007a). The interdomain linker connecting both domains may have a role in positioning of the two domains to each other to provide an appropriate conformation for substrate binding. The plant specific elongation of the interdomain linker sequence in AtStr1 was suggested to provide an extended hydrophobic environment surrounding the substrate binding site, enabling the protein to bind substrates as large as proteins. If this is true, the plant two-domain Str might

act in the regulation of other proteins by the direct transfer of sulfane sulfur (Burow et al., 2002; Bartels et al., 2007a).

Analysis of three dimensional Str structures

The tertiary structure of bovine rhodanese is composed of two domains which, in spite of a negligible sequence homology, are characterized by very similar three-dimensional folds connected by a loop at the surface of the molecule. Each domain displays α/β topology, with a central parallel five-stranded β-sheet surrounded by α-helices on both sides. The structural similarity of the two rhodanese domains has been considered as the prototype of divergent evolution from a common ancestor protein, which, after gene duplication and under the constraint of tertiary structure conservation, led to the almost complete obliteration of sequence similarity between the N- and the C-terminal halves (Russel et al., 1978; Bordo et al., 2000).

The *Azotobacter* RhdA protein was crystallized and its structure solved (Bordo et al., 2000). In spite of a strong similarity of an overall conserved protein in comparison to bovine rhodanese, the crystallographic investigations show that the process of substrate recognition in *Azotobacter* RhdA is based on a widely mutated active-site environment. The activity is essentially dependent on the main-chain conformation of the active-site loop and on the effect of an ensuing positive electrostatic field on the pK_a of the catalytic residue C230. Side-chains from the active-site loop, as well as from the surrounding regions, are supposed to be important for the process of substrate selectivity (Bordo et al., 2000). The crystal structure of 3-MP Str from *E. coli*, the SseA protein, displays conformational variation of the rhodanese active site loop, hosting the catalytic Cys residue. This structure may support a new sulfur transfer mechanism involving C237 as the nucleophilic species and H66, R102 and R262 as residues assisting catalysis (Spallarossa et al., 2004).

The 3-MP Str from *Leishmania major* (*L. major*) is a crescent-shaped molecule comprising three domains. The N-terminal and central domains are similar to the TS Str and create the active site containing a persulfurated catalytic C253 and an inhibitory sulfite coordinated by R74 and R185. A serine protease-like triad, comprising D61, H75, and S255, is near C253 and represents a conserved feature that distinguishes 3-MP Str from TS Str. During catalysis, S255 may polarize the carbonyl group of 3-MP to assist thiophilic attack, whereas R74 and R185 bind the carboxylate group. The *L. major* 3-MP Str is unusual with an 80-amino acid

C-terminal domain, bearing remarkable structural similarity to the FK506-binding protein class of peptidylprolyl cis/trans-isomerase. This domain may be involved in mediating protein folding and Str-protein interactions (Alphey et al., 2003).

The three-dimensional structure of the *Arabidopsis* protein encoded by At5g66040 (AtStr16) was determined by nuclear magnetic resonance (NMR) spectroscopy. AtStr16 contains a central β-sheet flanked on both sides by α-helices. The striking distinctive feature of the AtStr16 structure is an extra β-hairpin connecting the β1α1β2α2β3β4 and α3β7α4β8α5β9 which may play an important role in binding a specific substrate (Cornilescu et al., 2006). The three-dimensional structure of the rhodanese homology domain of At4g01050 (amino acid 175-295) (AtStr4) from *Arabidopsis* was also determined. Conventional sequence alignment did not display significant homology with proteins of known structure or function. But more sensitive algorithms provided evidence that this sequence may have a rhodanese fold. Structural analyses by NMR show a small α/β domain with a central five-stranded β-sheet surrounded by four α-helices (Pantoja-Uceda et al., 2005). For *Arabidopsis* AtStr proteins more work to analyze the three-dimensional structure by NMR or X-ray crystallography has to be done, especially elucidation of the two-domain AtStr including the substrate or a substrate analogue.

Determination of in vitro activities of Str

A number of molecules can serve as sulfur donors in the Str reaction, such as TS, thiosulfonates, persulfides, and 3-MP. Several compounds, such as cyanide, thiols, and dithiols, can act as acceptors (Fig. 3). The term sulfane sulfur designates sulfur atoms that are bonded covalently in chains only to other sulfur atoms. Examples are the outer sulfur of TS ($^{-}SO_3S^{-}$) and thiosulfonate ions (RSO_3S^{-}), the internal chain sulfurs of organic and inorganic polysulfides (RSS_nSR), where R represents an anion or organic group, persulfides (RSS^{-}), polythionates ($^{-}O_3SS_nSO_3^{-}$), and elemental sulfur (S_8) (Wood, 1987).

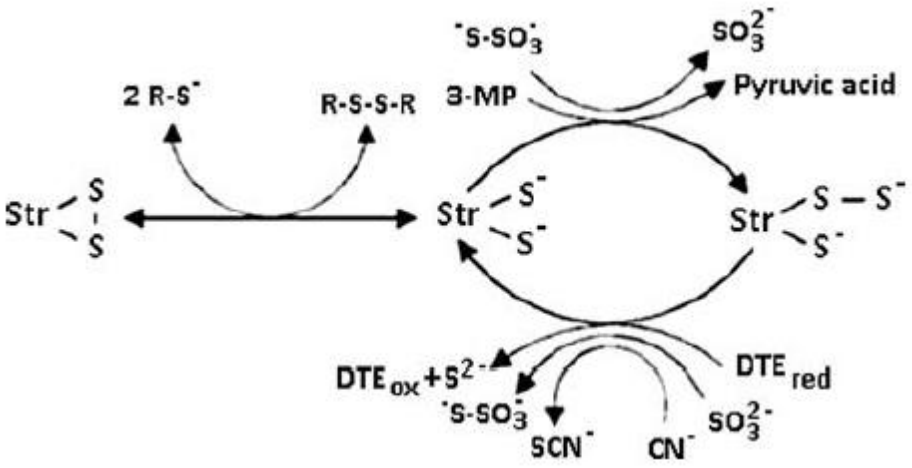


Figure 3. Reaction scheme for 3-MP Str and TS Str. 3-MP, 3-mercaptopyruvate; DTE, dithioerythritol (modified from Papenbrock and Schmidt, 2000a).

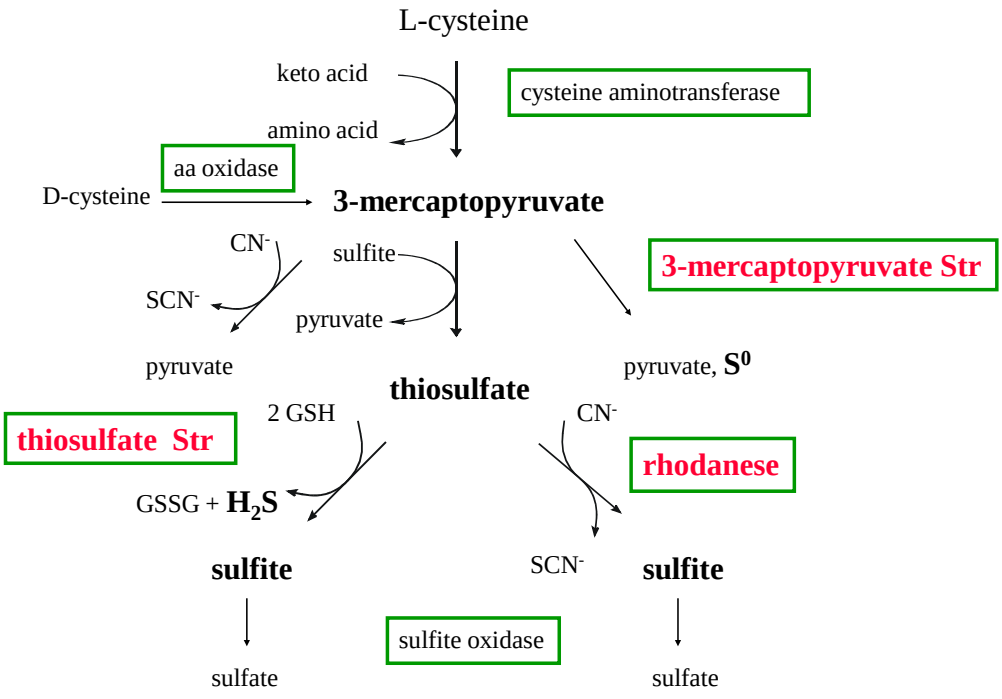


Figure 4. Putative reactions catalyzed by Str.

Most scientists use the enzyme assay described by Sörbo (1955). In the meantime, a number of alternative assays were successfully developed: the analysis of thiocyanate by capillary electrophoresis has been introduced (Glatz et al., 1999), a continuous assay was described based on the continuous determination of the sulfite product (Cannella et al., 1984), and recently the very sensitive ^{1}H-NMR assay was developed (Melino et al., 2003). Some ideas exist about the nature of enzymes which synthesize the substrates for Str, such as 3-MP and TS. To understand the function of an enzyme in the metabolic network of the cell the enzymes around the protein of interest have to be identified. For example, in the case of 3-MP Str in plants one has to postulate enzymes which catalyze the synthesis of 3-MP (Fig. 4). In animals 3-MP is presumably formed by transamination of Cys; Cys transaminase could catalyze the formation of L-glutamate and 3-MP from 2-oxoglutarate and L-Cys. Theoretically, 3-MP could also be synthesized from L-Cys by parallel release of H_2O_2 and ammonium catalyzed by an amino acid oxidase or by a Cys dehydrogenase releasing 3-MP, ammonium and protons. In any case 3-MP is a derivative of Cys and production of 3-MP might also be involved in regulation of the Cys pool. Up to now, most of the metabolic pathways suggested still remain hypothetical and more work needs to be done (Westley, 1973; White, 1982; Huang et al., 1998).

Beside a high homology of several short motifs in Str even the positions of single amino acids are conserved in all species investigated. It was assumed that these amino acids are relevant for the specificity of substrate binding. The role of non-Cys residues with respect to either 3-MP or TS specificity was investigated by mutagenesis studies (Luo and Horowitz, 1994; Nagahara et al., 1995; Nagahara and Nishino, 1996). A number of amino acids are indispensable for substrate binding and substrate specificity for 3-MP or TS, respectively. In both Str enzymes, 3-MP Str and rhodanese, from rat at least two amino acids in the active site are conserved, both enzymes accept 3-MP and TS but the ratio of their respective enzyme activities differs. C247 represents the catalytic site (formation of a persulfide) and R187 the substrate binding site. R187 and R196 of rat 3-MP Str are critical residues in determining substrate specificity for 3-MP, while R185, R247, and K248 of rat rhodanese are critical residues in determining substrate specificity for TS.

The purified as well as the recombinant rat 3-MP Str reveals K_m values for 3-MP in the low millimolar range. This K_m of the rat 3-MP Str for 3-MP is quite high, but also the k_{cat} is high; therefore 3-MP Str could be active at low physiological concentrations of 3-MP. 3-MP Str deficiency in the inherited human disease mercaptolactate-Cys disulfidurea results in

alternative metabolism of 3-MP (Nagahara et al., 1995; Nagahara and Nishino, 1996). K_m values for 3-MP of 0.2 mM have been reported for 3-MP Str from *L. major* using a thiol as the sulfur acceptor substrate (Williams et al., 2003). The 3-MP Str from *E. coli* (SseA) has a high affinity for 3-MP with a K_m value of 5 µM (Colnaghi et al., 2001). These observations support a role for 3-MP as the *in vivo* substrate for these 3-MP Str.

The reaction mechanism of rhodanese follows a ping-pong pattern (Westley and Heyse, 1971). The reaction mechanism for 3-MP Str was reported to follow a sequential pattern based on the results of steady state kinetics (Jarabak and Westley, 1978; Nagahara and Nishino, 1996). It is still an open question whether the donor and acceptor substrates enter the active site in order.

Also the formation of selenium-substituted rhodanese by reaction with selenite and glutathione was shown. A selenium-bound rhodanese could be used as the selenium donor by reaction with selenite and glutathione in the *in vitro* selenophosphate synthetase assay. Selenophosphate is the active selenium-donor compound required by bacteria and mammals for the specific synthesis of SeCys-tRNA, the precursor of seleno-Cys in selenoenzymes (Ogasawara et al., 2001).

The two-domain AtStr resemble the 3-MP Str from mammals. In both proteins the residues which are important for binding 3-MP are conserved while putative residues necessary for TS binding have been replaced during evolution (Nakamura et al., 2000; Papenbrock and Schmidt, 2000a; 2000b). Due to the fact that AtStr1 has to activated by a thiol, such as 2-mercaptoethanol (Fig. 3), it was speculated that reduced sulfur is bound to both Cys residues, C332 and C339, and both sulfur atoms are involved in the reaction mechanism. The replacement of each Cys residue resulted in mutant forms which differed significantly in their stability, in their specific Str activities, and in their kinetic parameters which were determined for 3-MP as well as for TS as sulfur substrates: Mutation of the putative active site Cys (C332) essentially abolished activity; for C339 a crucial role at least for the turnover of TS could be delineated (Burow et al., 2002; Bartels et al., 2007a). The function of C339 awaits further clarification in the future. The comparison of the kinetic parameters revealed that the specific activity of AtStr1 was much higher when 3-MP was used as sulfur donor in comparison to TS. However, the K_m value for TS was much lower and finally the k_{cat}/K_m value was higher than with 3-MP. Both substrates could be metabolized naturally, but the physiological levels of 3-MP are rather low in comparison to the K_m determined *in vitro*

(Papenbrock and Schmidt, 2000a); the kinetic data may therefore indicate that better substrates still need to be found (Burow et al., 2002).

Mobilization of the sulfur of Cys as persulfide is the first step of sulfur transfer into thiamine, molybdopterin, 4-thiouridine, biotin and lipoic acid, but the pathways diverge completely. For the first three compounds, one or several proteinic persulfides are involved, ending in the nucleophilic attack of a sulfur, persulfide, sulfide or thiocarboxylate on a carbonyl equivalent (Schievelbein et al., 1969). ThiI, a protein originally suggested to be involved in thiamine biosynthesis (Thi operon), is an enzyme common to the biosynthetic pathways leading to both thiamine and 4-thiouridine in tRNA. The *E. coli* enzyme contains a C-terminal extension displaying sequence similarity to rhodanese. The C456 of ThiI aligns with the active site Cys residue of rhodanese; mutation impaired Str activity and the generation of 4-thiouridine in tRNA Only the ThiI proteins from *E. coli* and *H. influenza* possess the sequence of limited similarity to rhodanese (Donadio et al., 1990; Palenchar et al., 2000).
One of the Str (AtStr12) is annotated as peptidyl-prolyl cis-trans isomerase-like protein. However, to our knowledge no experimental evidence for this enzyme activity neither *in vitro* nor *in vivo* has been demonstrated.
Recently, a reaction chain consisting of three enzymes was shown to be involved in sulfide oxidation in animals. A membrane-bound sulfide: quinone oxidoreductase converts sulfide to persulfides and transfers the electrons to the ubiquinone pool. Subsequently, a sulfur dioxygenase in the mitochondrial matrix oxidizes one persulfide molecule to sulfite, consuming molecular oxygen. The final reaction is catalyzed by a Str, which adds a second persulfide from the sulfide: quinone oxidoreductase to sulfite, resulting in the final product TS. This role in sulfide oxidation is an additional physiological function of the mitochondrial Str in animals (Hildebrandt and Grieshaber, 2008). The plant Str might be involved in a similar reaction chain producing TS in its reverse reaction. However, so far the other partners have not been identified in plant mitochondria.

The labile sulfane sulfur atom has been shown to have effects in biochemical systems which suggests that it may have several regulatory functions (Toohey, 1989; Wróbel et al., 2009). This conclusion is supported by evidence that sulfane sulfur is generated by partially known metabolic pathways, that carrier proteins for stabilizing and transporting are widely distributed, and that it is effective *in vitro* at very low concentration in regulating the activities of many enzymes. Its properties of a very high potency and short half-life are consistent with

a role as a finely-tuned regulator (Toohey, 1989).

A role in redox homeostasis: Interaction of Str and thioredoxins

Several lines of evidence support the hypothesis that Str are involved in the maintenance of redox homeostasis by interacting with thioredoxins. First results came from enzyme activity measurements in different species. The recombinant mammalian rhodanese catalyzes the direct oxidation of reduced thioredoxin evidently by reactive oxygen species. It was suggested that at least one Str isoform is involved in the detoxification of intramitochondrial oxygen free radicals (Nandi et al., 2000). Kinetic analysis revealed that catalysis by purified GlpE from *E. coli* occurs by way of an enzyme-sulfur intermediate utilizing a double-displacement mechanism requiring an active-site Cys. The K_m for TS was determined to 78 mM, the K_m for cyanide to 17 mM. When thioredoxin was used as acceptor the K_m was only 34 µM when TS was near its K_m, suggesting that thioredoxin or related dithiol proteins could be physiological substrates for Str (Ray et al., 2000).

Recently, the rat Str was suggested to be involved in the maintenance of the redox homeostasis by interacting with thioredoxin. Oxidative stress decreased 3-MP Str activity and increased the amount of Cys, a precursor of thioredoxin or glutathione. Furthermore, these cellular reductants restore the activity. Thus the redox state regulates 3-MP Str activity at the enzymatic level, and on the other hand, 3-MP Str controls redox to maintain cellular redox homeostasis. As an intermediate the very stable formation of Cys-sulfenate was shown having even a lower redox potential than glutathione (Nagahara and Katayama, 2005). In the same rat system Cys32 of *E coli* thioredoxin reacted with two redox-active Cys of rat 3-MP Str by forming an intersubunit disulfide bond and a sulfenyl Cys247. A consecutively formed disulfide bond between thioredoxin and 3-MP Str must be cleaved for the activation. *E. coli* C32S thioredoxin however, did not activate 3-MP Str. Reduced thioredoxin turns on a redox switch for the enzymatic activation of 3-MP Str which contributes to the maintenance of cellular redox homeostasis in rat cells (Nagahara et al., 2007).

Also in mammalian cells interaction of Str and thioredoxin plays an important role in the balance of the metabolism. The organo-sulfane sulfur compound, sodium 2-propenyl TS, was found to induce inhibition of Str activity in tumor cells. The activity of the enzyme was restored by thioredoxin in a concentration- and time-dependent manner. The results suggest an involvement of the thioredoxin-thioredoxin reductase system in cancer cell cytotoxicity by

organo-sulfane sulfur compounds and highlight the correlation between apoptosis induced by these compounds and the damage to the mitochondrial enzymes involved in the repair of the Fe-S cluster and in the detoxification system (Sabelli et al., 2008).

The increasing knowledge about redox regulation in plants shows more and more its importance in maintenance of the metabolism. In recent years the efforts to identify the major players in redox regulation by different methods are tremendous, including the characterization of thioredoxins. In a proteomic affinity approach an interaction of thioredoxin with 50 mitochondrial proteins was found, among them AtStr1 (Balmer et al., 2004). In a comparative proteomic approach cytosolic thioredoxin h3 from *Arabidopsis* was used in three methods for the identifcation of interacting proteins (Marchand et al., 2006). All together 73 interacting proteins could be identified, however, none of the cytoplasmic Str. All together more than 40 thioredoxins and thioredoxin-like proteins were identified in *Arabidopsis* (Meyer et al., 2005). Since a long time it is known that plants possess two thioredoxin systems, a cytoplasmic system including several thioredoxins and an NADPH-dependent thioredoxin reductase, and a specific chloroplastic system characterized by a ferredoxin-dependent thioredoxin reductase. Recently, also a functional plant mitochondrial system could be identified consisting of two thioredoxins and two NADPH-dependent thioredoxin reductases with dual-targeting (Laloi et al., 2001). As was discussed above also Str are localized in these compartments.

Spectroscopic studies using AtStr1 und mutants thereof suggest also a larger peptide or protein as substrate (Bartels et al., 2007a). Based on these enumerated results an investigation to analyze the interaction of several *Arabidopsis* thioredoxins with a number of Str by bimolecular fluorescence complementation (BiFC) (Bracha-Drori et al., 2004; Walter et al., 2004) was started. Specific interactions among a mitochondrial thioredoxin with mitochondrial AtStr1, among a plastidic thioredoxin with plastidic AtStr16, and a cytosolic thioredoxin with cytosolic AtStr2 was demonstrated (Holtgrefe and Papenbrock, unpublished results). It is still an open question how the specificity of the interactions among thioredoxins and target proteins can be explained. More informations about the redox interactome are needed. In the background of published results one can assume that Str might act as a thioredoxin peroxidase with the formation of a sulfenate at the active-site Cys. Therefore plant Str might play a role in the control of redox homeostasis in the different subcellular compartments (Papenbrock et al., 2009).

For several Str the in planta function is still not clear

Despite the presence of Str activity in many living organisms, the physiological role of these enzymes has not yet been established unambiguously. While in mammals Str may be involved in the elimination of toxic cyanogenic compounds (Vennesland et al., 1982; Nagahara et al., 1999) its ubiquity suggests additional physiological functions. It has been proposed that Str detoxify oxygen radicals, e.g. by acting as a thioredoxin oxidase in mitochondria (Nandi et al., 2000), are involved in sulfate assimilation (Donadio et al., 1990), transport specific sulfur compounds (Laudenbach et al., 1991), and may act as a sulfur invertase in the formation of prosthetic groups in Fe-S cluster proteins, such as ferredoxin (Bonomi et al., 1977; Pagani et al., 1984).

The biosynthesis of several vitamins, enzymes and cofactors includes a step of sulfur transfer and the incorporation of sulfur into the respective substrate molecule. This could be carried out by Str as was shown for the biosynthesis of thiamine and thiouridine in *E. coli* (Palenchar et al., 2000) and for the synthesis of the molybdenum cofactor in *E. coli* and humans (Leimkühler and Rajagopalan, 2001; Matthies et al., 2004). Especially one-domain Str proteins have been associated with specific stress conditions (Bordo and Bork, 2002). In general, Str might further activate or deactivate distinct proteins by direct transfer of sulfane sulfur and thus fulfill a regulatory role in the organism (Toohey, 1989).

One of the *Arabidopsis* proteins containing one rhodanese domain is involved in the biosynthesis of the molybdenum cofactor synthesis (AtStr13, Fig. 2). Four plant enzymes depend on molybdenum: nitrate reductase, sulfite oxidase, xanthine dehydrogenase, and aldehyde oxidase. In order to gain biological activity and fulfill its function in enzymes, molybdenum has to be complexed by a pterin compound thus forming the molybdenum cofactor. Molybdenum cofactor synthesis protein 3 (AtStr13/Cnx5) is essential during biosynthesis of the molybdenum cofactor. After molybdopterin synthase has transferred two sulfur atoms to precursor Z, it has to be resulfurated in order to reactivate the enzyme for the next reaction cycle of precursor Z conversion. This resulfuration is catalyzed by AtStr13/Cnx5 involving an adenylation of molybdopterin synthase followed by sulfur transfer (Matthies et al., 2004). AtStr13/Cnx5 is a two-domain protein consisting of an N-terminal domain responsible for adenylating molybdopterin synthase and a C-terminal rhodanese domain where the sulfur is bound to a conserved Cys in the form of persulfide (Matthies et al., 2004; Mendel, 2007).

The detoxification of cyanide was suggested as the main function of Str in plants. The widespread occurrence of ethylene synthesis suggests that this pathway is the principle source of endogenous cyanide in many plants although cyanide can be produced by other metabolic processes, e.g. hydrolysis of cyanogenic glycosides and in amino acid oxidase reactions (Kende, 1993). Ethylene is produced during ripening of fruits and also during senescence processes in leaves (Abeles, 1973). During ethylene biosynthesis, one molecule of cyanide is produced for each molecule of ethylene by the oxidation of 1-aminocyclopropane-1-carboxylic acid in the presence of iron and oxygen (Blumenthal et al., 1968; Manning, 1988). 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 protein families could act in its detoxification, Str and *β*-cyano-L-alanine synthase (CAS, EC 4.4.1.9). Str catalyze the formation of the less toxic thiocyanate. In mammals this compound is mainly excreted in urine (Nagahara et al., 1999); for plants thiocyanate-degrading enzymes are unknown although they have to be postulated. CAS catalyzes the formation of *β*-cyano-L-alanine from Cys in the presence of cyanide (Blumenthal et al., 1968). *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). A number of experiments have been done to prove the hypothetical involvement of Str in cyanide detoxification in plants. Thus far, the evidence for a role of plant Str in cyanide detoxification is rather low: Str activity was detected in the same order of magnitude in cyanogenic and non-cyanogenic plants (Chew, 1973) The results were confirmed in the way that no correlation between cyanogenesis and rhodanese activity was found (Kakes and Hakvoort, 1992). An involvement of AtStr1 in cyanide detoxification as in animals appears rather unlikely since the expression level of *AtStr1* and Str activity in total protein extracts remained unchanged in cyanide treated plants (Meyer et al., 2003).

A function in providing reduced sulfur for Fe-S cluster was investigated with contradictory results. Pagani and coworkers demonstrated the transfer of reduced sulfur into the Fe-S cluster of ferredoxin in spinach (Pagani et al., 1984). Whereas the preliminary analysis of an *atstr1* T-DNA insertion mutant showed that the mutation had no effect on the activity levels of Fe-S cluster containing proteins, suggesting that AtStr1 is not directly involved in Fe-S cluster assembly (Nakamura et al., 2000). However, Fe-S cluster are very sensitive to oxidative stress. Therefore decreasing oxidative stress by regulation of redox homeostasis as

thioredoxin peroxidase could also protect Fe-S cluster stability.

In radish and tobacco dark-inducible, senescence-associated homologues of AtStr15 encoded by *Rsdin1* and *Ntdin*, respectively, were identified that accumulated upon prolonged darkness, ethylene, cytokinin and heat stress (Azumi and Watanabe, 1991; Shimada et al., 1998; Yang et al., 2003). Due to similarity to this proteins most group VI AtStr have been annotated in the databases as senescence-associated proteins. The proteins share similarity to the C-terminal domain of AtStr1, to the GlpE protein in *E. coli* (Ray et al., 2000) as well as to other distinct stress-related proteins such as sulfide dehydrogenase from *Wolinella succinogenes* (Klimmek et al., 1999), phage-shock protein PspE from *E. coli* (Adams et al., 2002), and to the hsp67B2 from *Drosophila* (Shimada et al., 1998).

In agreement with the database annotations the expression of *AtStr15*, *AtStr16*, and *AtStr18* as well as Str activity in total protein extracts have been shown to increase with progressing age (Bartels et al., 2007b). In contrast, the expression of *AtStr14* decreased in five-week-old *Arabidopsis* plants and also at low sulfate supply, indicating a distinct role in sulfur metabolism. While the 3-MP Str activity in total protein extracts increased continuously with progressing age of the plants, the TS Str activity was strongly induced from week five to the end of the experiment after six weeks. The expression patterns of *AtStr16* and *AtStr18* as well as that of the senescence-associated *SAG13*, used as a marker for senescence in a previous work (Meyer et al., 2003), paralleled with the pattern of TS Str activity (Bartels et al., 2007b). AtStr16 (At5g66040) and AtStr18 (At5g66170) have been identified as TS Str proteins (Bauer and Papenbrock, 2002) and are likely to play a role in senescence.

The most interesting and by far best characterized of the six proteins in group VI is AtStr15 (AtSEN1). The senescence-associated *AtStr15* gene is induced commonly during senescence caused by several senescence inducing factors including age, darkness, and phytohormones as well as by inoculation with diverse pathogens (Oh et al., 1996; Chung et al., 1997; Weaver et al., 1998; Schenk et al., 2005). *AtStr15* expression was clearly induced at nutritional stress conditions (low sulfate or phosphate, added TS) and in darkness, and increased with age (Bartels et al., 2007b). In contrast to the results obtained by Bartels et al. (2007b), the expression of *AtStr15* was slightly reduced in leaves and roots at low phosphate in a recent microarray analysis (Misson et al., 2005). Despite all these information the biological function of the *AtStr15* gene product could not be determined yet. In tobacco, a homologue of AtStr15, Ntdin1, was recently shown to be involved in molybdenum cofactor biosynthesis (Yang et al., 2003). According to results from Bartels (2006) a role of AtStr15 in molybdenum

cofactor biosynthesis could be excluded. This was confirmed by recent results on the function of AtStr15/AtSEN1 (Schenk et al., 2005). Taking together the information obtained on AtStr15/AtSEN1 thus far, the protein may have a function in general stress response of the plant caused by various biotic and abiotic triggers. AtStr13 (At5g55130) in group V was shown to act as molybdopterin synthase sulfurylase (Mendel, 2007). The AtStr12/SIR1 protein is composed of a rhodanese-like domain and a ubiquitin-activating enzyme E1-like domain. SIR1 is a regulator of many auxin-inducible genes. The *sir1* mutant was resistant to sirtinol, a small molecule activating many auxin-inducible genes, and displays auxin-related developmental phenotypes. An involvement of the protein in the propagation of auxin-signals in *Arabidopsis* was suggested (Zhao et al., 2003).

Another putative activity of a rhodanese-containing *Arabidopsis* protein was found by chance. Arsenate reductase (AR) activity was determined in an arsenate-hyperaccumulating fern. The reaction mechanism was very similar to the previously reported activity of Acr2p from yeast, using glutathione as the electron donor. A T-DNA knockout mutant of *Arabidopsis* with disruption in the putative Acr2 gene had no AR activity (Duan et al., 2005). According to the nomenclature by Bartels et al. (2007b) the orthologous protein in *Arabidopsis* corresponds to AtStr5 (At5g03455), a nuclear protein with a Cys in the active center. So far the AR activity could not be confirmed for the recombinant *Arabidopsis* protein (Papenbrock, unpublished results). Interestingly, the same protein was shown to act as Cdc25 dual-specificity tyrosine-phosphatase (Landrieu et al., 2004a). Therefore, further experiments are needed to clarify the cellular function of AtStr5.

In a proteome-wide characterization of seed aging, it was shown that a loss in seed vigor is associated with a decreased level of 3-MP Str, highlighting further the importance of sulfur metabolism and homeostasis in seeds. As yet unknown important role(s) of Str in seed physiology and quality is predicted (Rajjou et al., 2008).

Str might be involved in defence mechanisms against pathogens

It is known since antiquity that sulfur has protective effects against pests and diseases. Sulfur supply thus influences plant resistance. In a recent study the increased disease susceptibility of sulfur deficient *Brassica napus* plants towards distinct fungal and bacterial pathogens was shown to be caused at least partially by a reduction of sulfur-dependent phytoanticipins (Dubuis

et al., 2005). Elemental sulfur, glutathione, sulfite, H_2S, and TS can be products of specific Str reactions. Besides other sulfur containing compounds like Cys-rich antifungal proteins, glucosinolates and phytoalexins, these substances might play important roles in plant disease resistance (Cooper et al., 1996; Williams et al., 2002; Cooper and Williams, 2004; Williams and Cooper, 2004; Rausch and Wachter, 2005). There are hints that Str proteins might also play a role in pathogen defence reactions of the plant. In a suppression subtractive hybridization of non-infected and with *Verticillium dahlia* infected tomato plants a tomato Str homologue to AtStr1 could be isolated (Jonathan Howarth, Rothamsted, UK, personal communication). In a differential display analysis aiming to isolate genes related to resistance towards the powdery mildew fungus *Erysiphe graminis* (*E. graminis*) in wheat (*Triticum aestivum* L.) a Str gene displaying similarity to AtStr1 was identified that might be involved in pathogen resistance against *E. graminis* in wheat (Niu et al., 2002). A rhodanese-like protein displaying similarity to AtStr17 was identified in phloem exudates of *Curcubita maxima* Duch. by two-dimensional gel electrophoresis and subsequent mass spectrometry. The protein is suggested to be involved in stress and defence responses of the plant by acting as phytohormone and/or in signalling (Walz et al., 2004). Since Str proteins have been proposed to detoxify reactive oxygen species in mammals (Nandi et al., 2000) one could speculate about a possible protective function of the AtStr proteins during pathogen defence by protecting healthy tissue from oxidative damage, again by regulation of redox homeostasis as thioredoxin peroxidase.

Are Str a relict in evolution?

Some authors consider rhodanese as a biochemical relict of a time when the earth's atmosphere contained large amounts of hydrogen cyanide (Pagani et al., 1987). But this is a rather pessimistic view of such an ubiquitous enzyme (Hama et al., 1994). It is thought, on the basis of the amino acid sequence evolution around the catalytic Cys that the C-terminal catalytically active domain of prokaryotic TS Str is an ancestral protein of the rhodanese family and the duplicated molecule is an 3-MP Str precursor (Nagahara, 2007). A change of 3-MP Str occurred in the oxidizing atmosphere of the earth after the appearance of cyanobacteria. In this process a leucine residue was replaced with a Cys residue to regulate cellular redox homeostasis in the new oxidizing earth atmosphere. Because plants evolved under reducing conditions, there is a leucine residue at the redox-sensing switch. In a phylogenetic evolution life forms acquired the redox-sensing switch in 3-MP Str in adaptation

to the oxidizing atmosphere in the earth (Nagahara, 2007).

CONCLUSIONS

Str belong to a very exciting protein family and we are just at the beginning to elucidate their functions in the organism. It was shown already that Str are involved in regulation of metabolism including protein biosynthesis, co-factor biosynthesis, protection against biotic and abiotic stress, regulation of redox homeostasis, seed development among others. Probably from the current systematic analysis of *Arabidopsis* mutants we will be able to learn about many more functions in plants and maybe also in other organisms.

CHAPTER 3

SULFURTRANSFERASE AND THIOREDOXIN SPECIFICALLY INTERACT AS DEMONSTRATED BY BIMOLECULAR FLUORESCENCE COMPLEMENTATION ANALYSIS AND BIOCHEMICAL TESTS

Abstract

Sulfurtransferases (Str) and thioredoxins (Trx) are members of large protein families. Trx is a disulfide reductase and plays an important role in redox-related cellular processes. It interacts with a broad range of proteins. Str catalyze the transfer of a sulfur atom from a suitable sulfur donor to nucleophilic sulfur acceptors *in vitro*, but the physiological roles of these enzymes are not well defined. Several studies in different organisms demonstrate a protein-protein interaction of Str with members of the Trx family. We are interested to investigate the specificity of the interaction between Str and Trx isoforms. In order to use the bimolecular fluorescence complementation (BiFC), several Str were cloned into pUC-SPYNE and different Trx into pUC-SPYCE split-YFP vector. Both plasmids containing the sequences for the putative interaction partners were transformed into *Arabidopsis* protoplasts and screened using a confocal laser scanning microscope. Compartment- and partner-specific interactions could be observed in transformed protoplasts. Replacement of cysteine residues in the redox site of Trx abolished the interaction signal. Therefore the redox site is not only involved for the redox reaction but also responsible for the interaction with partner proteins. Biochemical assays support a specific interaction among Str and Trx. Based on the results obtained the interaction of Str and Trx indicates a role of Str in the maintenance of the cellular redox homeostasis.

INTRODUCTION

Rhodanese activity, the transfer of a reduced sulfane sulfur from thiosulfate to cyanide, was found in many organisms. The main function of these rhodanese/sulfurtransferase (Str) proteins in the organism was described as cyanide detoxification (Westley, 1973). Later bioinformatic analysis demonstrated that proteins containing a Str/rhodanese domain are present in all three domains of life (Bordo and Bork, 2002). In addition, in most organisms protein families with a large number of members have been identified. This high abundance of Str sequences makes the sole function as cyanide detoxification enzymes questionable. The protein family of Str in *Arabidopsis thaliana* consists of 20 proteins containing one or two rhodanese domains and are located in different cellular compartments (Papenbrock et al., 2010). For some of the recombinant Str proteins from *Arabidopsis* (AtStr) an *in vitro* substrate specificity could be shown, either for thiosulfate (TS) or for 3-mercaptopyruvate (3-MP). However, the function of most Str in the plant organism remains unresolved so far. In recent years the results of several independent research approaches indicate that Str interact specifically with different proteins (Caplan et al., 2008; Aussignargues et al., 2012), mainly with thioredoxins (Trx), regulatory disulfide proteins (Nandi et al., 2000; Nagahara et al., 2007; Sabelli et al., 2008). All together more than 40 Trx and Trx-like proteins were identified in *Arabidopsis* containing a five amino acid redox site with two cysteine residues (WCXXC) and also located in different compartments of the cell. Unlike animal and yeast counterparts, the function of Trx in plant mitochondria is largely unknown (Meyer at al., 2005). There are two major questions Trx researchers ask: why are there so many Trx and Trx-like proteins found in plants in comparison to other taxa and what is the biochemical basis for their target protein specificity (Lee et al., 2012). Therefore more information about the redox interactome is needed.

First results for a Str/Trx interaction came from enzyme activity measurements in different species. Bacterial and mammalian Str from mitochondria catalyze the direct oxidation of reduced Trx demonstrating that Trx could be a physiological substrate for Str (Nandi and Westley, 1998; Ray et al., 2000). In animals at least one Str/rhodanese isoform could serve in the detoxification of intramitochondrial oxygen free radicals (Nandi et al., 2000). The redox state regulates 3-MP Str activity from rat at the enzymatic level and 3-MP Str maintains the cellular redox homeostasis (Nagahara and Katayama, 2005). This hypothesis was supported by a proteomic affinity approach demonstrating an interaction of Trx with 50 mitochondrial

proteins, among them AtStr1 (Balmer et al., 2004), and similar approaches (Peltier et al., 2004). In the background of published results one can speculate that Str might act as a Trx peroxidase with the formation of a sulfenate at the active site cysteine. Cysteine-sulfenate is very stable and has even a lower redox potential than glutathione (Nagahara and Katayama, 2005; Nagahara et al., 2007).

To postulate a functioning redox cycle of Str and Trx, the latter has to be first reduced and then oxidized again by a Trx reductase as indicated in the scheme shown in Figure 1. There exists a family of six NADPH-Trx reductases in *Arabidopsis* (Meyer et al., 2005). In the plastids three out of four proteins are ferredoxin-dependent Trx reductases, in the cytoplasm and the mitochondria the proteins are NADPH-dependent Trx reductases (Laloi et al., 2001). The molecular basis for the Trx specificity, if there is any, of Trx reductases is not known yet.

The goal of this study is to find specific interactions among Str and Trx proteins and to elucidate the molecular basis thereof. Several principally different methods were applied to investigate this postulated interaction. They are based on close physical proximity as a prerequisite in bimolecular fluorescence complementation (BiFC) as discussed in Bracha-Drori et al. (2004) and Walter et al. (2004). In the biochemical tests the interaction could either be a protein-protein interaction or a loose substrate chanelling interaction. Results indicate a compartment- and partner-specific protein-protein interaction for some Str/Trx pairs combined with a more unspecific substrate-chanelling type of reaction. The results will help to identify some *in vivo* function of Str proteins in plants.

MATERIAL AND METHODS

Chemicals

All chemicals were obtained from Roth (Karlsruhe, Germany), Sigma (Taufkirchen, Germany) or Applichem (Darmstadt, Germany) if not otherwise mentioned.

Growth and harvest of plants

Seeds of *Arabidopsis thaliana* (L.) Heynh., ecotype Columbia, were originally obtained from the Arabidopsis stock center at the Ohio State University. After two days of stratification seeds were sown on soil and grown for six weeks in a climate chamber under following conditions: Short day period with a quantum rate of 120 µmol m^{-2} s^{-1} (sodium vapor lamp, SON-T Agro 400, Philips) and 21°C.

Isolation of protoplasts

The lower epidermises of about ten leaves of *Arabidopsis* plants were peeled off with a piece of glue strip (Prof. Dr. R. Scheibe, Osnabrück, Germany, unpublished). With the peeled surface the leaves were put into a petri dish with enzyme buffer I (0.4 M mannitol, 20 mM KCl, 20 mM MES, 10 mM $CaCl_2$, 0.1% (w/v) bovine serum albumin, pH 5.7). Buffer I was removed with a pasteur pipette and buffer II [1.5% (w/v) cellulase Onozuka R-10 (Serva, Heidelberg, Germany) and 0.4% macerocyme R-10 (Serva) dissolved in buffer I] was added. The leaves were incubated for 40 min at 25°C and slow orbital shaking (30 rpm). The protoplast suspension without the leaves was centrifuged at 100*g* for 3 min. The pellet was washed carefully with W5 buffer (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES (pH 5.7), 5 mM glucose). After removal of the buffer the pellet was resuspended in 6 ml W5 buffer. Incubation for 30 min on ice followed by a centrifugation for 1 min at 4°C at 100*g*. The supernatant was discarded and the pellet resuspended in MMG buffer (9.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES, pH 5.7) and incubated on ice for 20 min.

Transformation of protoplasts

Eighty µg plasmid DNA of Str and Trx, respectively, were transformed into the protoplasts with the following method. The plasmid DNA was put carefully in 200 µl protoplast suspension. Stepwise 220 µl PEG buffer [40% PEG 4000 (Merck, Darmstadt, Germany), 0.2 M mannitol, 100 mM $CaCl_2$] was pipetted slowly and carefully to the protoplasts and incubated for 15 min at room temperature (RT). After adding 0.5 ml, 1 ml, 2 ml, and 4 ml, respectively, W5 buffer, the solution was mixed carefully and incubated for 15 min at RT. An overnight incubation in darkness followed. The supernatant was discarded till only a small amount was left. The protoplasts were investigated under a confocal laser scanning microscope (CLSM) (LSM 510 Meta, Zeiss, Jena, Germany).

Cloning procedures

The plasmids pUC-SPYNE (4,616 bp) and pUC-SPYCE (4,832 bp) (abbreviation for split YFP N-terminal/C-terminal fragment expression) used are described (Walter et al., 2004). All primer pairs and templates are summarized in Table 1. When necessary cDNA was prepared by RT-PCR using a kit (MBI Fermentas, St. Leon-Roth, Germany).

For the wild-type Str and Trx the PCR tubes contained 0.2 mM dNTPs, 0.4 µM of each primer (Eurofins MWG Operon, Ebersberg, Germany), 0.25 µl Dream *Taq* DNA polymerase (MBI Fermentas), 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 60 s at 94°C, 60 s at the different annealing temperatures required, and 60 s at 72°C. The process was finished with an elongation phase of 600 s at 72°C. The amplified PCR fragments were ligated into pGEM-T, sequenced and then ligated into the YFP containing vectors pUC-SPYNE or pUC-SPYCE and introduced into the *Escherichia coli (E. coli)* strain XL1-blue.

For the production of the mutated Trx two PCRs were done. The initial PCR contained 0.2 mM dNTPs, 0.5 µM of each primer (Eurofins MWG Operon), 0.5 µl Phusion DNA polymerase (MBI Fermentas) and about 1 µg template DNA in a final volume of 50 µl. Before starting the first PCR cycle the DNA was denatured for 30 s at 98°C followed by 28 cycles conducted for 10 s at 98°C, 30 s at 54°C and 30 s at 72°C. The process was finished

with an elongation phase of 600 s at 72°C. The PCR products were used as the template for the final PCR. The PCR contained 0.2 mM dNTPs, 0.5 µM of each primer (Eurofins MWG Operon), 0.5 µl Phusion DNA polymerase (MBI Fermentas) in a final volume of 50 µl. Before starting the first PCR cycle, the DNA was denatured for 30 s at 98°C. Initially five cycles were run, each consisted of denaturation at 98°C for 10 s, followed by annealing at 49°C for 60 s and finally extension at 72°C for 30 s. Then, 23 cycles were run. Each of these cycles consisted of denaturation at 98°C for 10 s, followed by annealing at 54°C for 30 s and finally extension at 72°C for 30 s. A final extension was done at 72°C for 10 min. The amplified PCR products were ligated into pJET (MBI Fermentas), sequenced, ligated into the pUC-SPYCE and then introduced into the *E. coli* strain XL1-blue.

All clones produced and/or used for BiFC analysis during this study are summarized in Table 1. In general all Str sequences were ligated into the pUC-SPYNE vector and the Trx sequences into the pUC-SPYCE vector. As control the transcription factor T14-3c, a 14-3-3 transcription factor protein with a very high interaction potential, was used (Walter et al., 2004).

The Str expression clones in pQE-30 AtStr1 and a mutant thereof AtStr_C_{339}S, AtStr2, AtStr14, AtStr15, AtStr16, and AtStr18 were produced during previous studies (Papenbrock & Schmidt, 2000a, 2000b; Bauer & Papenbrock, 2002; Burow et al., 2002). The Trx expression clones of Trxo1, Trxy1, Trxh1, and Trxh3 in pET16b were produced using the primers listed in Table 1 in which the primer of Trxh3 and Trxy1 were used for pUC-clones and expression clones. The *E. coli* Trx (accession number AAC76786) was purchased from Sigma (T-0910). A clone expressing NADPH-dependent Trx reductase from *Triticum aestivum* (TaNTR, accession number AJ421947, 34.9 kDa) was obtained (Serrato et al., 2002).

Table 1. The table includes the names of the proteins, AGI numbers, primer sequences with internal primer number. The underlined bases were the exchanged ones for the mutation. Number of base pairs amplified by PCR and the molecular mass of the encoded proteins.

Name	AGI no.	Primer	Bp	MW [kDa]
AtStr1	At1g79230	332 tctagaatggcctcgaccctt	950	41.90
		333 ctcgagtgaagaagattcaac		
AtStr2	At1g16460	480 tctagaatggcttcttctgga	950	34.71
		481 ctcgagtgaagaagaacccac		
AtStr14	At4g27700	482 tctagaatggcttcacttact	700	2487
		483 ctcgaggtcttcttcaattgt		
AtStr15	At4g35770	484 tctagaatggaaaccactgct	549	20.04
		485 ctcgagctcttctaccggcag		
AtStr16	At5g66040	336 ggatccatggcggaggagagcaga	423	12.68
		337 ctcgagagcctttgtaggaag		
AtStr18	At5g66170	486 tctagaatgtctcaatcaatc	411	14.71
		487 ctcgagattagcagatggctc		
Trxy1	At1g76760	338 tctagaatggcgtcaatttct	516	19.25
		339 ctcgagtggcttcacttttag		
Trxy1_C_{93}S		338 tctagaatggcgtcaatttct	516	19.23
		339 ctcgagtggcttcacttttag		
		634 gcaacctggtctggtccttgc		
		635 gcaaggaccagaccaggttgc		
Trxy1_C_{96}S		338 tctagaatggcgtcaatttct	516	19.23
		339 ctcgagtggcttcacttttag		
		636 tgtggtcctagccagttcatg		
		637 catgaactggctaggaccaca		

Table 1. continued

Name	AGI no.	Primer	Bp	MW [kDa]
Trxy1_$C_{93}S$_$C_{96}S$		338 tctagaatggcgtcaatttct	516	19.22
		339 ctcgagtggcttcacttttag		
		638 acctggtctggtcctagccag		
		639 ctggctaggaccagaccaggt		
TPTrxy1		338 tctagaatggcgtcaatttct	186	6.67
		691 ttcttccgaagcacgtcgaggagt		
Trxm1	At1g03680	ccgctcgagcaagaatttgttgatgctggttgc	537	19.67
		gctctagaatggctgcttacacgtgtacttc		
Trxm4	At3g15360	ccgctcgagctcgaccaagaatctttctatag	579	21.17
		gctctagaatggcgtcgttactcgattcc		
Trxf1	At3g02730	gctctagaatggctggagttgtgcgat	534	19.33
		ccgctcgagacttgatgcagctggtttg		
Lilium5	At4g26160	gctctagaatggctggagttgtgcgat	663	24.35
		ccgctcgagacttgatgcagctggtttg		
Trxo1	At2g35010	656 tctagaaagggaaattggtcg	585	21.19
		657 ctcgagtcacttgtagagctg		
		454 ggatccaagggaaattggtcg		
		455 aagcttcttgtagagctgttc		
Trxo1_$C_{118}S$		656 tctagaaagggaaattggtcg	585	21.17
		657 ctcgagtcacttgtagagctg		
		658 cgcctggtctggaccatgcag		
		659 ctgcatggtccagaccaggcg		
Trxo1_$C_{121}S$		656 tctagaaagggaaattggtcg	585	21.17
		657 ctcgagtcacttgtagagctg		
		660 gtgtggaccatccaggtttatctc		
		661 gagataaacctggatggtccacac		

Table 1. continued

Name	AGI no.	Primer	Bp	MW [kDa]
Trxo1_$C_{118}S$_$C_{121}S$		656 tctagaaagggaaattggtcg	585	21.16
		657 ctcgagtcacttgtagagctg		
		662 gcctggtctggaccatccaggttt		
Trxh1	At3g51030	663 aaacctggatggtccagaccaggc	342	12.67
		664 tctagagcttcggaagaagga		
		665 ctcgagttaagccaagtgttt		
		ggaattccatatggcttcggaagaaggacaagtg		
		cgggatccttaagccaagtgtttggcaatg		
Trxh1_$C_{40}S$		664 tctagagcttcggaagaagga	342	12.66
		665 ctcgagttaagccaagtgttt		
		666 gcttcttggtctggaccatgtc		
		667 gacatggtccagaccaagaagc		
Trxh1_$C_{43}S$		664 tctagagcttcggaagaagga	342	12.66
		665 ctcgagttaagccaagtgttt		
		668 tgtggaccatctcgtttcatc		
Trxh1_$C_{40}S$_$C_{43}S$		669 gatgaaacgagatggtccacac	342	12.64
		664 tctagagcttcggaagaagga		
		665 ctcgagttaagccaagtgttt		
		670 cttggtctggaccatctcgtttc		
Trxh1withTPTrxy1		671 gaaacgagatggtccagaccaag	528	19.34
		338 tctagaatggcgtcaatttct		
		665 ctcgagttaagccaagtgttt		
Trxh3	At5g42980	ggaattccatatggccgcagaaggagaagttat	354	13.11
		cgggatcctcaagcagcagcaacaactg		

Plasmid DNA purification

To obtain high amounts of plasmid DNA of the Str and Trx containing plasmids the Maxi Kit from Qiagen (Hilden, Germany) was used with a following phenol/chloroform (1:1) extraction and sodium acetate (3 M, pH 5.3) (Baker, B.-V.-Deventer, Netherlands) precipitation.

Expression and purification of recombinant proteins

AtStr1, AtStr1_C_{339}S, AtStr2, AtStr14, AtStr16, AtStr18, Trxy1, Trxh1, Tryh3, Trxo1, and TaNTRB 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 containing 100 µg ml^{-1} ampicillin). The induction was carried out for 3 h with 0.5 mM final concentration of isopropyl-L-D-galactoside at 30°C. Cell lysis was obtained by adding lysozyme (final concentration 1 mg ml^{-1}) and vigorous homogenizing using an ultrasonic cell disruptor (Branson Ultraschall, Dietzenbach, Germany). The recombinant proteins were purified under non-denaturing conditions by affinity chromatography with nickel affinity resin and by using the Äkta Prime Plus Chromatography system (GE Healthcare, Freiburg, Germany). For buffer exchange the Äkta Prime Plus Chromatography system was also used with a column containing Sephadex G-25 Superfine crosslinked with dextran beads. The desalted proteins could be used directly for the enzyme tests. The purity of the protein preparations was checked by SDS-PAGE (Laemmli, 1970) and subsequent Coomassie staining.

Sulfurtransferase activity

Before the recombinant Str proteins were used in the interaction assay the activity was tested with the Str activity assay as described (Papenbrock and Schmidt, 2000a).

Activity test for thioredoxins by an insulin assay

The activity of the Trx was tested by the insulin assay described by Holmgren, (1979). Stock solutions of insulin were prepared with 10 mg ml^{-1} (1.67 mM) by suspending 50 mg insulin

(Sigma) in 4 ml of 0.05 M Tris/HCl, pH 8.0, and adjusting to pH 2 to 3 by addition of 1.0 M HCl and rapidly titrating the solution back to 8.0 with 1.0 M NaOH followed by a pH meter. Finally, the volume was adjusted to 5 ml with water. Freshly prepared solution of insulin, 1 mg ml^{-1} in 0.1 M potassium phosphate (pH 6.5) and 2 mM EDTA, was stored on ice. The assay mixture was prepared by addition of 250 µl insulin plus 1.5 µM Trx and water to give a final volume of 300 µl. The reaction was started by pipetting 2 µl 100 mM DTT in all reaction tubes, except the blank. As control the assay was done without Trx. The measurements were performed at 650 nm using a microplate detection instrument (Synergy Mx, BioTek, Bad Friedrichshall, Germany) with no further mixing of the plate for assays lasting up to 80 min.

Biochemical analysis of the interaction of Str and Trx by the H_2S releasing assay

Str activity was measured by the release of H_2S from 3-MP in the presence of Trx. The assay contained in a total volume of 300 µl: 100 mM Tris/HCl pH 8.0, 0.025 µM AtStr1 or mutant AtStr1_$C_{339}S$, alternatively 1 µM of AtStr2, AtStr14, AtStr16, or AtStr18, 4 µM of recombinant Trx proteins, 0.5 µM NADPH-dependent Trx reductase, 50 µM NADPH, 1 µM tris-(2-carboxyl)-phosphine (TCEP) (Sigma), and 50 µM 3-MP as starting reagent. For the pre-incubation buffer, Str, Trx, and TCEP were incubated for 30 min at 37°C. Then NADPH, NADPH-dependent Trx reductase and finally 3-MP were added. After an incubation for 20 min at 37°C the reaction was terminated by adding 30 µl of 30 mM $FeCl_3$ dissolved in 1.2 N HCl and 30 µl 20 mM *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride dissolved in 7.2 N HCl (Siegel, 1965). After 20 min incubation at RT the formation of methylene blue was determined at 670 nm. Solutions with different concentrations of Na_2S were prepared, treated in the same way as the assay samples, and were used for the quantification of enzymatically formed H_2S.

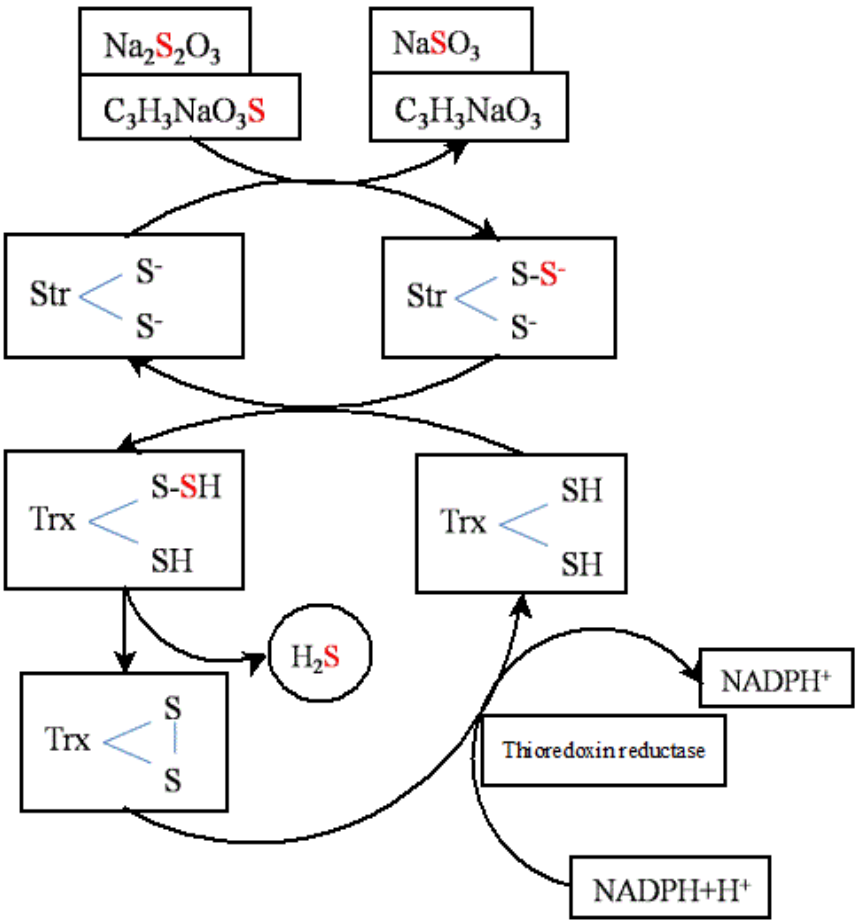


Figure 1. Schematic reaction cycle of Str, Trx and NADPH-dependent Trx reductase (modified after Nandi et al., 2000).

Cross-linking experiment

The experiments were done in 10 mM NaH_2PO_3 buffer at pH 7.0 in a final volume of 100 µl containing 10 µM of the respective protein and 1 mM bissulfosuccinimidyl suberate (BS3) for 1, 5, 10 and 30 min at 25°C. The reaction was stopped by addition of 15 µl gel loading buffer. SDS-PAGE was done according to Laemmli (1970) and stained with Coomassie brilliant blue.

Other procedures

Protein contents were determined according to Bradford (1976) using BSA as protein standard. Alignments were done with Clone Manager 9 (Sci-Ed, Cary, NC, USA) and with ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

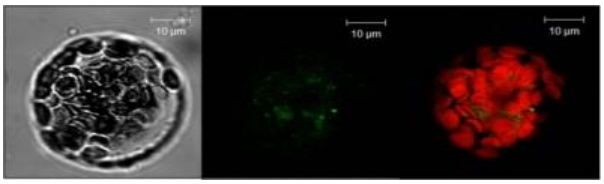
RESULTS

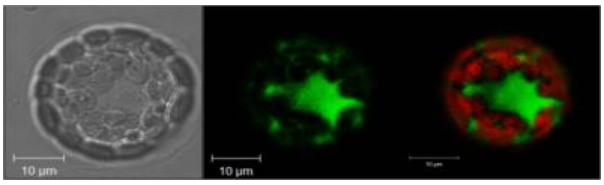
Sulfurtransferase interacts with thioredoxin as demonstrated by bimolecular fluorescence studies

Different combinations of Str and Trx in the vectors pUC-SPYNE and pUC-SPYCE, respectively, localized in the mitochondria (AtStr1, Trxo1), in the cytoplasm (AtStr2, AtStr18, Trxh1, Trxh3) or in the plastids (AtStr14, AtStr15, AtStr16, Trxy1, Trxm1, Trxm4, Trxf1, Lil5) and mutations thereof were transiently transformed into protoplasts isolated from *Arabidopsis* leaves. The co-transformed protoplasts were analyzed using a CLSM. Bright field images, YFP emission and overlayed autofluorescence of chlorophyll and YFP emission were taken (Fig. 2). As control for the experimental set up two parts of the tobacco 14-3-3 transcription factor protein T14-3c coupled to YFP were used (Walter et al., 2004) and a clear signal in the nucleus was visible (data not shown). Although it is not possible to make a number-based quantification of signal intensity in YFP split experiments the abundance of transformed protoplasts and the intensity of the fluorescence signals can be estimated by screening many transformation events. Based on this screening process the interactions among the partners were classified as weak, intermediate and strong (Table 2). Among all protein pairs investigated in this study the fluorescence of the T14-3c protein was the highest observed (data not shown).

All positive interaction pairs were localized in the same compartment as was previously shown or predicted for the single proteins (Bauer et al., 2004; Meyer et al., 2005). The interaction of Str and Trx is compartment-specific. Each Str tested interacted with at least one Trx localized in the same compartment (mitochondrion: AtStr1/Trxo1; cytoplasm: AtStr2/Trxh1, AtStr18/Trxh3; plastid: AtStr14/Trxf1; AtStr16/Trxy1). Only AtStr15 showed a positive signal with several Trx in the plastid (Trxy1, Trxm1, Trxf1, Lilium5). We are aware that interaction partners are transported into the respective compartments due to their target sequence (mitochondria, plastid) or remain in the cytoplasm. Therefore the cytoplasmic Trxh1 was fused with the transit peptide of Trxy1 but neither with the cytoplasmic AtStr2 nor with the plastidic AtStr15 a positive signal was obtained, although the cytoplasmic Trxh1 usually shows a very strong interaction with AtStr2. Except Trxm4, all Trx proteins investigated interacted with at least one AtStr. Trxm4 did not show any interaction with the offered plastidic proteins AtStr14, AtStr15 and AtStr16. Including their transit peptides Trxm1 and

Trxm4 share only 46% identify and 65% similarity. Their redox site is identical (WCGPC) but the features around the protein disulfides differ.


AtStr1/Trxo1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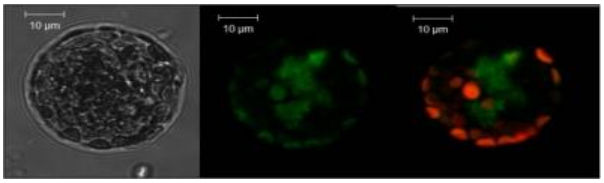
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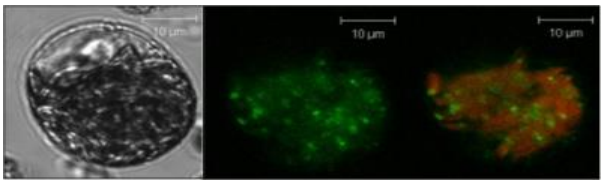
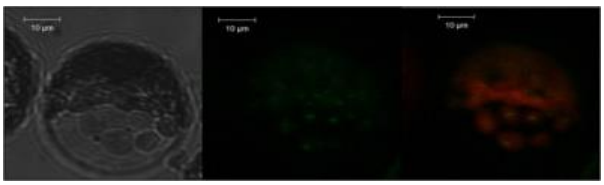
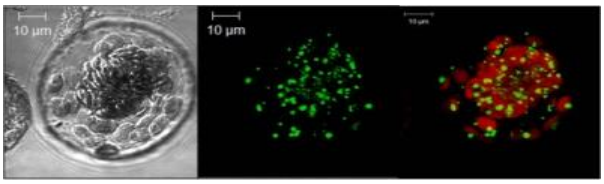
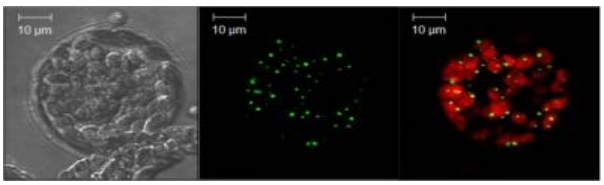
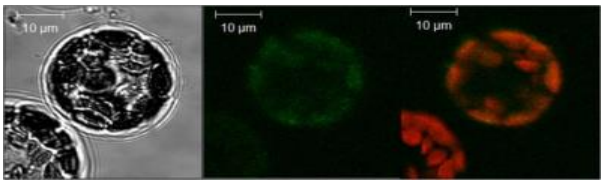

AtStr15/Lilium5


AtStr15/Trxf1


AtStr15/Trxm1


AtStr15/Trxy1


AtStr16/Trxy1

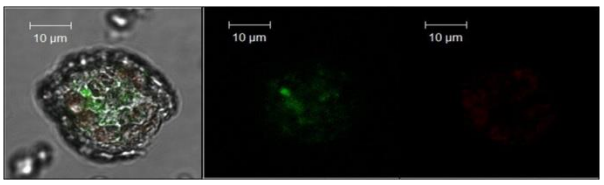

AtStr18/Trxh3

Figure 2. Exemplary results of positive BIFC interaction. The interactions of AtStr15/Trxf1, AtStr16/Trxy1 and AtStr18/Trxh3 were classified as weak, of AtStr1/Trxo1, AtStr14/Trxf1, and AtStr15/Lilium5 as intermediate, and of AtStr2/Trxh1, AtStr15/Trxm1, and AtStr15/Trxy1 as strong. Left: Bright field image, middle: YFP emission, right: overlay of autofluorescence and YFP emission.

Table 2. Summary of the results of BiFC analysis. + weak interaction, ++ intermediate interaction, +++ strong interaction among the partners. The division is based on the abundance of transformed protoplasts and the intensity of the fluorescence signals.

	AtStr1	**AtStr2**	**AtStr18**	**AtStr14**	**AtStr15**	**AtStr16**
Trxo1	++	-			-	
Trxo1_C_{118}S	-					
Trxo1_C_{121}S	-					
Trxo1_C_{118}S_C_{121}S	-					
Trxh1	-	+++	-		-	
Trxh1_C_{40}S		-				
Trxh1_C_{43}S		-				
Trxh1_C_{40}S_C_{43}S		-				
Trxh3		-	+		-	
Trxy1	-		-	-	+++	+
Trxy1_C_{93}S					-	
Trxy1_C_{96}S					-	
Trxy1_C_{93}S_C_{96}S					-	
TPTrxy1+Trxh1		-			-	
Trxm1				-	+++	-
Trxm4				-	-	-
Trxf1				++	+	-
Lilium5				-	++	-

Biologically significant protein-protein interactions are characterized by the involvement of essential amino acid residues in the contact zones of both interaction partners. Mutant variants that are affected in these critical residues might support the biological significance of the interaction. We assume that the cysteine residues in the redox site of Trx proteins mediate the contact among Str and Trx proteins. Therefore each and both of the respective cysteine residues in the redox site of Trxo1 Trxh1, and Trxy1 were replaced by serine residues (WCGPC: WSGPC/WCGPS/WSGPS).

The single replacements of the first cysteine residue Trxo1_C_{118}S, the second cysteine residue Trxo1_C_{121}S or of both cysteine residues Trxo1_C_{118}S_C_{121}S prevent an interaction with AtStr1. The respective amino acid replacements in Trxh1, Trxh1_C_{40}S, Trxh1_C_{43}S, and Trxh1_C_{40}S_C_{43}S, abolished the strong interaction with AtStr2. And also the strong interaction of Trxy1 with AtStr15 was completely abolished in all three mutant variants. The results clearly demonstrate that each cysteine residue is essential for the interaction with Str proteins. However, the residues are not the only feature for the specificity of the interaction based on the negative results of several plastidic Trx with one of the three Str proteins.

Enzyme activity of recombinant proteins

Based on the reaction scheme shown in Figure 1 the biochemical assays were designed to measure the produced H_2S. Before the purified recombinant proteins were used in combined assays, the single activities of the proteins were analyzed by independent biochemical reactions to demonstrate their intactness and correct folding. The activity of the recombinant Str proteins was measured by the formation of thiocyanate from 3-MP or TS in the presence of cyanide (Papenbrock and Schmidt, 2000a). The K_m values of both substrates is in the low millimolar range. However, it was found out that the H_2S releasing assay was not functioning at millimolar substrate concentrations and also when using TS as substrate. Therefore, thiocyanate formation was determined at different 3-MP concentrations (5 µM, 50 µM, 500 µM, 5 mM) to find a compromise between optimal substrate concentration for Str activity and technical limitations of the H_2S releasing assay (Fig. S1).

Finally, the best working concentration was set to 500 µM substrate (Fig. 3). Str activity of the two-domain Str proteins was higher with 3-MP, whereas the one-domain proteins showed higher activity with TS in agreement with published results (Bauer and Papenbrock, 2002)

(Fig. 3). Trx intactness was analyzed by the insulin assay (Holmgren, 1979). In short, in the presence of DTT Trx catalyzes the reduction of insulin disulfides. The rate of insulin reduction can be followed spectrophotometrically at 650 nm as turbidity formation from the precipitation of the free insulin B chain (Fig. 4). Five AtStr proteins could be expressed and purified as soluble proteins whereas several attempts and trials (expression, induction and purification conditions, different expression vector, purification under denaturing conditions and subsequent re-folding) to obtain active AtStr15 protein were not successful.

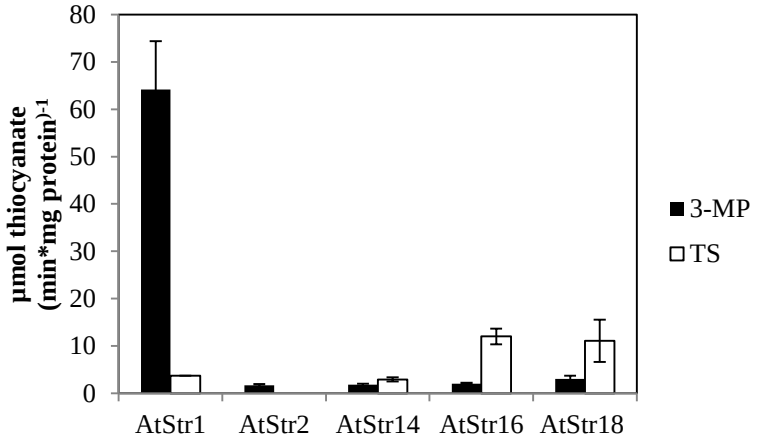


Figure 3. Sulfurtransferase activity of different recombinant Str proteins using 3-mercaptopyruvate (3-MP) (5 mM) and thiosulfate (TS) (5 mM) as substrates.

Four *Arabidopsis* Trx were expressed and purified. To investigate the specificity of the reaction partners in the assay and because the *E. coli* Trx has been used in previous experiments (Nandi and Westley, 1998; Nagahara et al., 2007), it was included in the analysis (Fig. 4). In the insulin activation assay Trxh3 showed the highest activity in comparison to Trxh1, Trxy1, and the *E. coli* Trx (Fig. 4) whereas Trxo1 was not active, also after several attempts to modify the expression conditions. In pre-tests the influence of the NADPH-dependent NTR species origin was analyzed. The origin and therefore sequence of the NTR, either from *E. coli* or from *T. aestivum,* did not influence the assay at all, although the *E. coli* enzyme is a selenocysteine protein with a molecular mass of 54.7 kDa and the *T. aestivum* protein has only 34.9 kDa (Serrato et al., 2002).

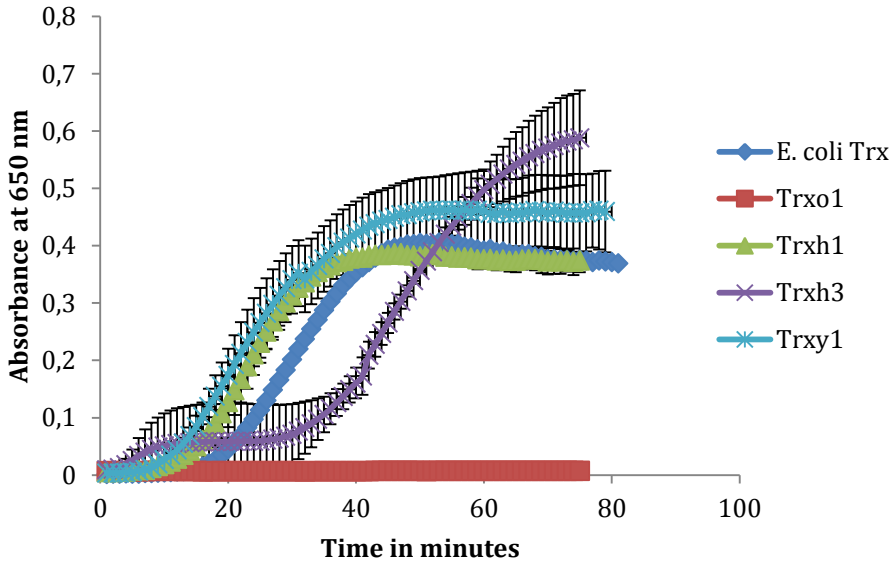


Figure 4. Activity test of recombinant Trx proteins demonstrated by their activation of insulin

All active AtStr and Trx proteins were used in the H_2S-releasing test according to the scheme shown in Figure 1. The activity of Str in the H_2S-releasing assay was tested at three different 3-MP concentrations: 10 µM (Fig. S2),50 µM (Fig. S3), and 500 µM (Fig. 3). As shown in Figure S4 and S5 the assay is very sensitive to 3-MP and the non-enzymatic activity without the presence of any Str or Trx protein is high (Fig. S5).

In general, the H_2S-releasing activity was highest in the presence of AtStr1 and all Trx proteins used. *E. coli* Trx did only increase the H_2S-releasing activity of AtStr1 but not of the other AtStr proteins. Trxh1 showed high activities with AtStr1, AtStr18, and AtStr14. Trxh3 increased slightly the activity of AtStr2 and AtStr18. Plastidic Trxy1 enhanced the H_2S-releasing activity of AtStr18. The mutant AtStr1_C_{339}S shows a reduced Str activity (Burow et al., 2002) and it was speculated that this indicates a binding site of a larger peptide or protein as substrate instead of small molecules such as TS or 3-MP (Bartels et al., 2007a). The mutant protein was used in the H_2S-releasing assay with Trxh3 as interaction partner. The activity was even higher than the wild-type activity of AtStr1 (data not shown). The cysteine residue C339 of AtStr1 does not play a role as binding partner of Trx.

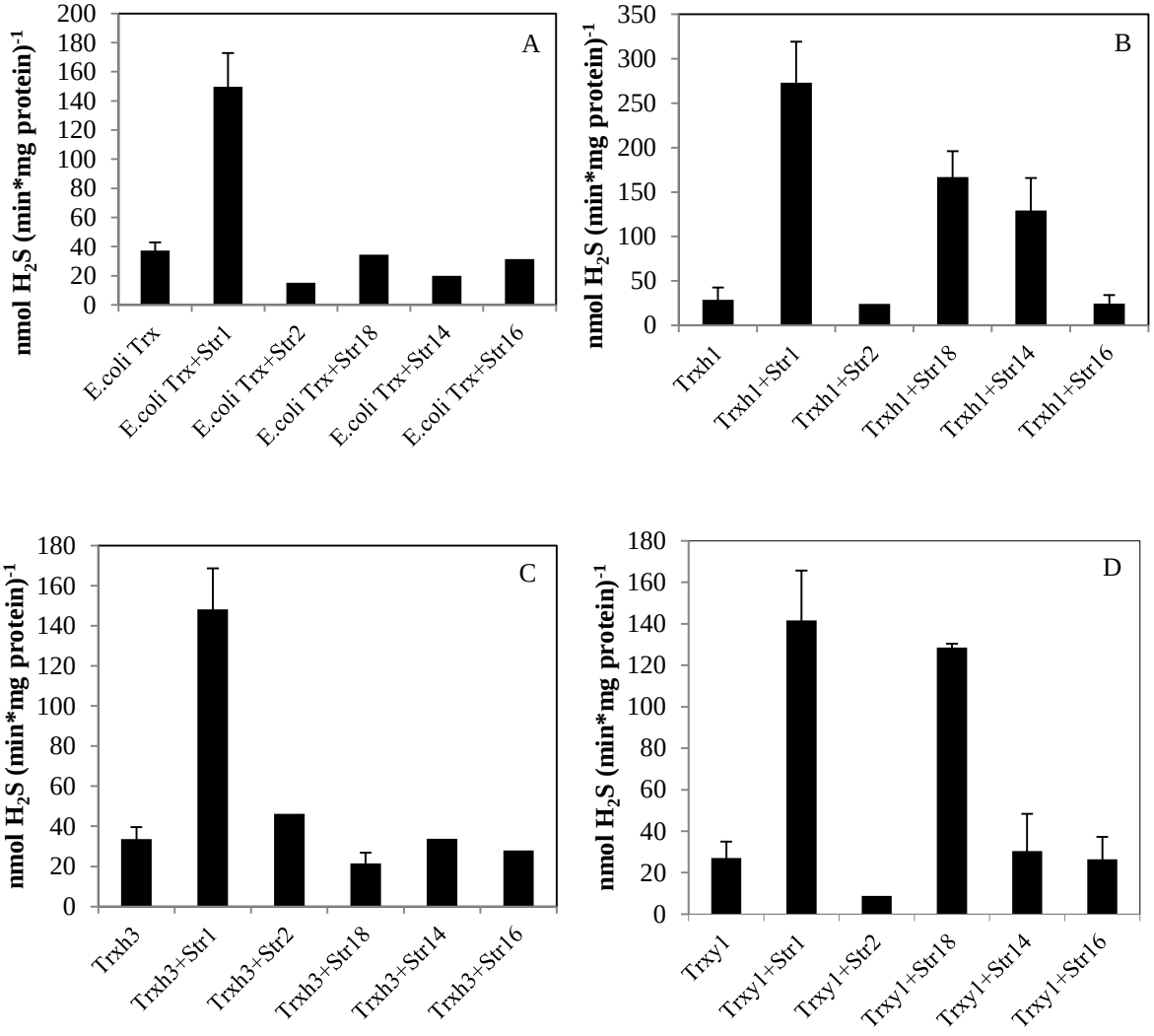


Figure 5. Combined enzyme assay using different recombinant Str and Trx proteins and 500 µM 3-MP as substrate. A) H_2S test of *E. coli* Trx and with five different Str. B) H_2S test of Trxh1 and five different Str. C) H_2S test of Trxh3 and five different Str. D) H_2S test of Trxy1 and five different Str.

The H_2S-releasing assay used for the analysis of the biochemical interaction of some AtStr and Trx proteins does not show any correlation with the results from the BiFC analysis. Another biochemical test was used by measuring the oxidation of NADPH (Nandi and Westley, 1998), with similar ambiguous results. Maybe the specificity of the interaction is based on protein compartimentalization in the cell or more suitable methods need to be applied.

Cross-linking

To complete the results of the interaction of Trx and Str cross-linking experiments with BS3 as cross-linker were done. Two different Str were tested with three different Trx. AtStr16 formed a complex with Trxy1 (Fig. 6A) and AtStr18 formed a complex with Trxh3 (Fig. 6C). No interaction was observed between AtStr16 and Trxh1. Also no interaction was visible between AtStr18 and Trxy1 or Trxh1. These results are in agreement with the BiFC analysis. A small band resulting from a complex formed between AtStr16 and Trxh1 might indicate a little interaction.

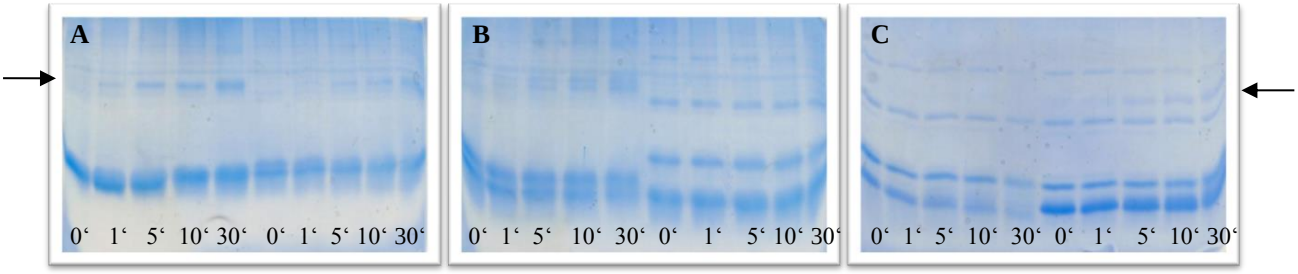


Figure 6. SDS gels of the cross-linking experiments. A) Lane 1-5 AtStr16 and Trxy1 after 0, 1, 5, 10 and 30 min incubation; lane 6-10 AtStr16 and Trxh1 after 0, 1, 5, 10 and 30 min incubation. B) Lane 1-5 AtStr16 and Trxh3; lane 6-10 AtStr18 and Trxy1. C) Lane 1-5 AtStr18 and Trxh1; lane 6-10 AtStr18 and Trxh3. The arrows showed the complexes.

DISCUSSION

Sulfurtransferase interacts with thioredoxin as shown by bimolecular fluorescence complementation studies

Several pairs of Str and Trx proteins from *Arabidopsis* showed interactions in split-YFP assays. BiFC significantly facilitate the visualization of the subcellular sites of protein interactions under conditions that closely reflect the normal physiological environment (Walter et al., 2004). All six AtStr proteins investigated, showed identical localization compartments and patterns using the co-transformed proteins in the BiFC analysis as of single transformation as GFP-coupled proteins (Bauer et al., 2004). This confirmation underlines the evidence of the BiFC interactions results. Even the association of AtStr15 with the thylakoid membrane supported by immunoelectron microscopy images (Bauer et al., 2004) were reproduced using split-YFP analysis (Fig. 2). The AtStr15 C-terminus contains a hydrophobic region of about 20 amino acids which is predicted to be transmembrane or membrane associated. Therefore, it was assumed that the protein is attached to the thylakoid membrane by the C-terminus. This spatial localization of AtStr15 could facilitate a protein-protein interaction close to membranes (Bauer et al., 2004).

The quantification of fluorescence signals in the BiFC assay remains difficult and the division into different interaction strength' as was done in this study is not statistically valid. At high expression levels also the free YFP fragments sometimes tend to associate non-specifically, thereby generating background fluorescence (Walter et al., 2004). BiFC fluorescence can reach only about 30% of the corresponding full length YFP. To overcome these shortages, the split-luciferase complementation approach was recently developed. It facilitates dynamic and quantitative *in vivo* analysis of protein interactions as the restoration of luciferase activity upon protein-protein interaction of investigated proteins is reversible at the initial state, complex formation can be competed by alternative interaction partners (Gehl et al., 2011). The application of this system for analyzing the Str/Trx interaction could verify the estimated intensity of the interactions.

We assume that negative complementation results are really the consequence of missing interaction of the Str/Trx pairs based in following evidence: The transient transformations were repeated several times over a long experimental period in different conditions by different scientists, always with the same results. In previous studies differences in the

expression of the two partners, demonstrated by YFP antibody experiments, have been detected, mainly when protein partners had significantly different molecular masses (Bracha-Drori et al., 2004; Walter et al., 2004). Here the differences are relatively small, especially among the one-domain Str and Trx. So far our results are not supported by other approaches for screening interaction partners of Trx proteins. In a comparative proteomic experiment, based on different biochemical traits for the identification of interacting proteins using cytosolic Trxh3, none of the cytoplasmic Str have been identified (Marchand et al., 2006). However, the complementarity and efficiency of the biochemical screening methods was low (Marchand et al., 2006). Therefore genetic methods or target-directed approaches might be more promising. We also used the database STRING which analyses protein-protein interactions based on several parameters (experimental, co-expression, genetic context, http://string-db.org/) However, neither for Str nor for Trx from *Arabidopsis* meaningful interaction partners were found.

With cross-linking experiments the interaction of Trx and Str was analyzed by another method. The experiments were performed with two different Str and three different Trx. In agreement with the results of the BiFC analysis, AtStr16 and Trxy1 as well as AtStr18 and Trxh3 formed a complex (Fig. 6A and 6C). A complex was detected in the experiment done with AtStr16 and Trxh1 (Fig. 6A). To confirm these results the cross-linking experiment has to be repeated. Furthermore the protein of AtStr18 was not clean enough, seen in the additional bands on the gel, so this experiment has to be repeated in any case.

Specificity of the interaction: The WCXXC redox site acts as a contact site

In the introduction we asked two question of more general interest, why is there such a high number of Trx proteins found in plants in comparison to other taxa and how is their target protein specificity determined. Both questions can be at least partially answered, mainly with the help of Trx mutants. The availability of several genomes of vascular and nonvascular plants allowed the establishment of a clear classification of the Trx genes and the chronology of their appearance during plant evolution. Putative Trx target proteins are involved in all aspects of plant growth, including basal metabolism, iron/sulfur cluster formation, development, adaptation to the environment, and stress responses (Meyer et al., 2012). During the evolution of green plants there was obviously a demand for many different, partially

highly specific Trx proteins with many different or with only a few interacting partners, revealing the high plasticity of the redox systems in plants.

Another aspect of the high abundance of members of the Trx family in plants is the differential expression. It was shown that Trxf and Trxm in *Pisum sativum* reduce plastidial fructose-1,6-bisphosphatase (FBPase) and malate dehydrogenase (MDH), respectively. Both Trx proteins show a different spatial pattern, as shown by immunocytochemistry. Whilst PsTRXm was localized to vascular tissues of all the organs analyzed (leaves, stems, and roots), PsTRXf was localized to more specific cells next to xylem vessels and vascular cambium (Traverso et al., 2008).

It is known that biologically significant protein-protein interactions are characterized by the involvement of essential amino acid residues in the contact zones of both interaction partners. Mutant variants that are affected in these critical residues and that result in loss of the interaction coincident with an altered plant phenotype are therefore suitable controls to verify the biological significance of a protein-protein interaction (Bhat et al., 2006). The mutant analysis of three Trx localized in different compartments fully supports the results obtained: Replacement of even one out of the two cysteine residues in the redox and putative contact site completely abolishes interaction.

The redox sites of the Trx proteins used in this study consist of the amino acids WCGPC in Trxf1, h1, m1, m4, o1, and y1, of WCGSC in Lilium5, and of WCPPC in Trxh3. We replaced only the cysteine residues in the redox site WCGPC but we assume that the replacement of the cysteine residues in the other redox sites leads to identical results, abolishment of interaction. One could also speculate that cysteine residues outside of the redox site are involved in the interaction. All Trx analyzed in this study contain more than two cysteine residues (plastidic: Trxy1, 4; Trxm1, 4; Trxm4, 3; Trxf1, 6; Lilium5, 6; mitochondrial: Trxo1, 3; cytosolic: Trxh1, 3; Trxh3, 3). However, the positions of the cysteine residues are not conserved and each single cysteine residue is distributed over the complete sequence as can be deduced from alignments of the respective protein sequences (data not shown).

Probably, amino acids in addition to the redox site are responsible for the specificity of the Trx-protein interaction. The structure of Trxh2 from barley (*Hordeum vulgare*) in a reaction intermediate complex with a protein substrate, barley alpha-amylase/subtilisin inhibitor (BASI), was reported. HvTrxh2 has the typical fold of Trx with a five-stranded β-sheet

surrounded by four α-helices in a βαβαβαββα topology. The overall structure of HvTrxh2 is representative for Trx proteins, as the Cα atoms can be superimposed on the crystal structures of oxidized Trx from other species. The crystal structure of this mixed disulfide shows a conserved spatially defined motif ($_{45}$WCGP$_{48}$-$_{87}$AMP$_{89}$-$_{104}$VGA$_{106}$) in HvTrxh2 interacting with a sequence of residues from BASI through van der Waals contacts and backbone-backbone hydrogen bonds. The observed structural complementarity suggests that the recognition of features around protein disulfides plays a major role in the specificity and protein disulfide reductase activity of HvTrxh2 (Maeda et al., 2006). We analyzed the hydrophobic motif in AtTrxh1 in comparison to the other Trx proteins used in this study (Table 3). Two Trx vary only in one amino acid in comparison to AtTrxh1, three in two, one in three and one in six amino acid residues. Therefore the specificity seems to be based only on a small number of amino acids in the Trx sequences. The counter part of the specificity criteria is based on the fold of the partner protein. BASI is recognized by HvTrxh2 primarily by a cysteine residue and two immediately preceding residues. This DWC is stabilized by the spatially defined motif, the substrate recognition motif, on the HvTrxh2 surface and three structural loops. In BASI a common right-handed disulfide bridge is formed between the cysteine residue and the cysteine residue in WCGP. Similar structures are probably found in Str proteins and other proteins interacting with Trx. Comparison with structurally related proteins shows that Trx shares a mechanism with glutaredoxin and glutathione transferase for correctly positioning substrate cysteine residues at the catalytic groups but possesses a unique structural element that allows recognition of protein disulfides (Maeda et al., 2006). Several non-Trx interaction partners of Str have been suggested. Probably other molecular mechanisms are responsible for the specificity. This has to be analyzed in the future in a similar elegant experimental set-up as used by Maeda et al. (2006).

Table 3. Motif of the redox site and substrate recognition site of the thioredoxins used in this study. Amino acids different from Trxh1 are highlighted in bold because this motif sequence was used by Maeda et al. (2006).

Thioredoxin	**Amino acid motif**
Trxo1	WCGPC-A**V**P-VGA
Trxh1	WCGPC-AMP-VGA
Trxh3	WC**P**PC-AMP-VGA
Trxy1	WCGPC-**AL**P-**E**GA
Trxm1	WCGPC-S**I**P-**I**GA
Trxm4	WCGPC-S**V**P-**I**GA
Trxf1	WCGPC-**V**VP-**T**GA
Lilium5	WCG**S**C-**VL**P-**CSL**
E. coli Trx	WCGPC-**GI**P-VGA

The cytoplasmic Trxh1 was fused with the transit peptide of Trxy1 but neither with the cytoplasmic AtStr2 nor with the plastidic AtStr15 positive signals were obtained (Table 2). The result supports that there is a specificity of protein pair interactions: Although AtStr15 reacts with four different plastidic Trx proteins it does not accept the newly designed cytoplasmic protein including the plastidic target peptide. One could assume that the overall three-dimensional structure of Trxh1 changes when an unusual transit peptide is attached and the substrate recognition loop motif is not correctly structured any more. Spectroscopic studies using AtStr1 und mutants thereof suggest also a larger peptide or protein as substrate instead of small molecules such as TS or 3-MP (Bartels et al., 2007).

Impact of the results about the putative function of Str in redox homeostasis

There are several indications that Str takes part in the maintenance of the redox homeostasis in cells in an interplay with Trx (Nagahara and Katayama, 2005). Str might act as a Trx (per)oxidase with the formation of a sulfenate at the active site cysteine (Nandi et al., 2000). Cysteine-sulfenate is very stable and has even a lower redox potential than glutathione (Nagahara and Katayama, 2005). In the rat system a cysteine of *E. coli* Trx reacted with two

redox-active cysteine residues of rat 3-MP Str by forming an intersubunit disulfide bond and a sulfenyl cysteine. A consecutively formed disulfide bond between Trx and 3-MP Str must be cleaved for the activation. The mutated *E. coli* Trx_C_{32}S however, did not activate 3-MP Str. Reduced Trx turns on a redox switch for the enzymatic activation of 3-MP Str which contributes to the maintenance of cellular redox homeostasis in rat cells (Nagahara et al., 2007). It was also shown that the rhodanese RhdA helps the nitrogen-fixating bacterium *Azotobacter vinelandii* in maintaining the cellular redox balance (Remelli et al., 2010). Our results from the BiFC analysis indicate that Str in a specific interaction with certain Trx proteins might play a role in the control of the redox homeostasis in each single subcellular compartments. Interestingly, regularly the deformation of protoplasts transformed with AtStr1 and Trxo1 was observed during the BiFC experiments. One could assume that the transformed mitochondria suffer under oxidative stress. It was suggested that Trx acts as a sensor and enables mitochondria to adjust key reactions in accord with prevailing redox state. By sensing redox in chloroplasts and mitochondria Trx enables the two organelles of photosynthetic tissues to communicate by means of a network of transportable metabolites. In this way, light absorbed and processed by means of chlorophyll can be perceived and function in regulating fundamental mitochondrial processes akin to its mode of action in chloroplasts (Balmer et al., 2004). For the plastidic Trxf1 and Trxm1 it is well known that they mediate light regulation of carbon metabolism through the activation of Calvin cycle enzymes. Recently, it was shown that both Trx are regulated by the transcription factor CCA1, part of the circadian clock, on the transcriptional level (Barajas-López et al., 2010), indicating circadian-controlled redox regulation.

Recently, it was demonstrated that 3-MP specific Str produces H_2S from 3-MP. Although a reducing substance is required for an intermediate persulfide at the active site of 3-MP Str to release H_2S, the substance has not been identified. Trx and dihydrolipoic acid (DHLA) associate with 3-MP Str to release H_2S. Other reducing substances, such as NADPH, NADH, GSH, cysteine and CoA, did not have any effect on the reaction. It was also shown that 3-MP Str produces H_2S from TS. The study provides a new insight into a mechanism for the production of H_2S by 3-MP Str (Mikami et al., 2011). Our biochemical assays support the *in vivo* possibility of the H_2S-release in the Str/Trx system. Therefore, in addition to the cellular redox homeostasis the production of H_2S as signal molecule and/or a toxic agent against microorganisms (Papenbrock et al., 2007) can be postulated.

Outview

The BiFC results show that the interaction between Str and Trx seems to be specific. Better biochemical assays have to be developed to characterize the specificity on the activity level. Additional methods such as gelfiltration of cross-linking approaches could be applied to overcome the high reactivity of 3-MP or sulfur-containing substrates in general. Completely, different methods could be used to investigate the specific interaction, such as the modified yeast two-hybrid system (Vignols et al., 2005) or Förster (fluorescence) resonance energy transfer (FRET) analysis. Based on the results obtained so far the interaction of Str and Trx indicates a role of Str in the maintenance of the cellular redox homeostasis. Screening of *trx*/*str Arabidopsis* double mutants have to proof this hypothesis.

APPENDIX

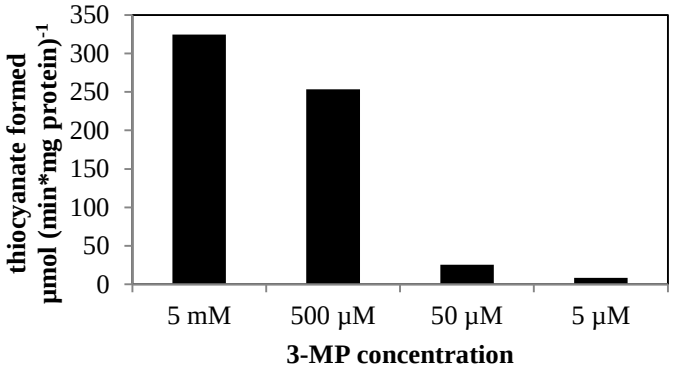


Figure S1. Str activity test of AtStr1 with different 3-MP concentrations.

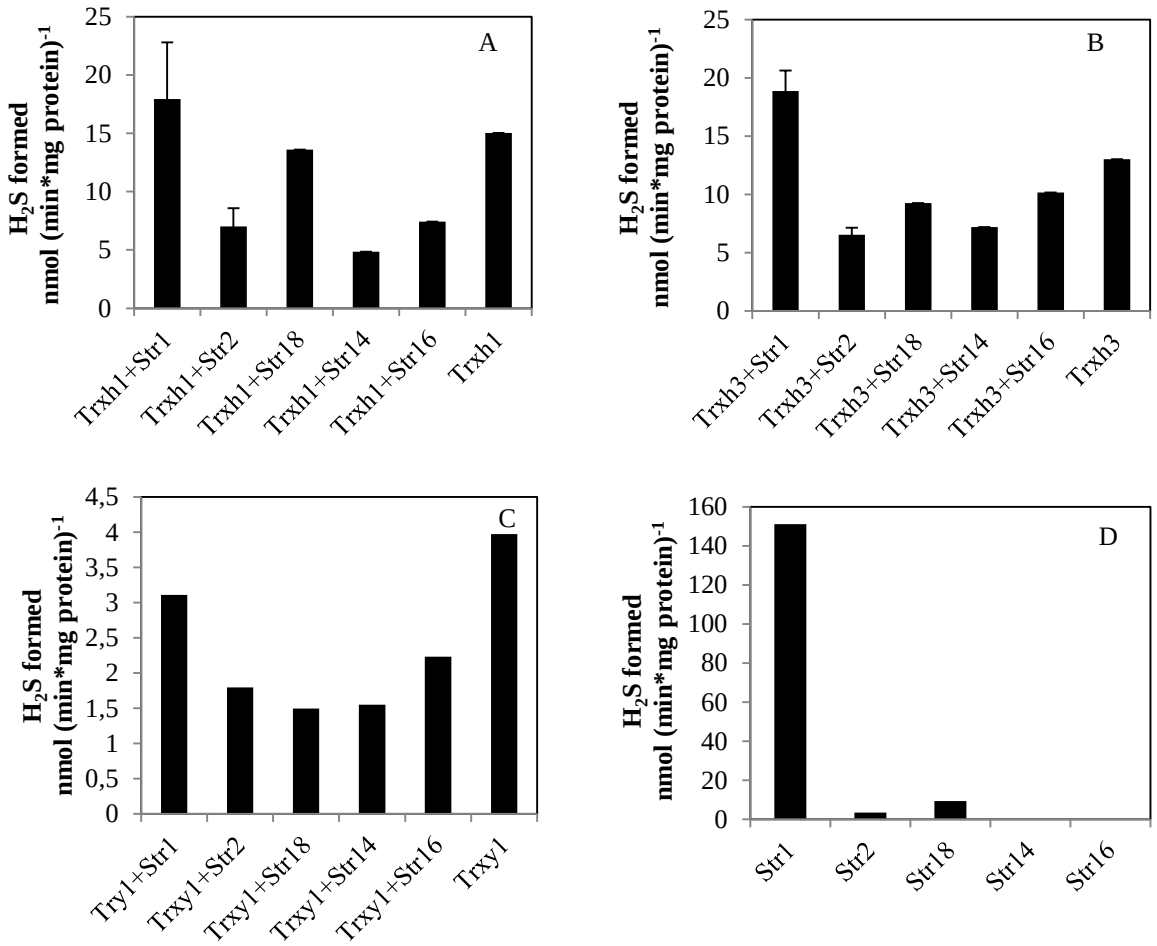


Figure S2. Combined enzyme assay using different Str and Trx and 10 µM 3-MP. A) H_2S test of Trxh1 with five different Str. B) H_2S test of Trxh3 with five different Str. C) H_2S test of Trxy1 with five different Str. D) H_2S test of five different Str without Trx.

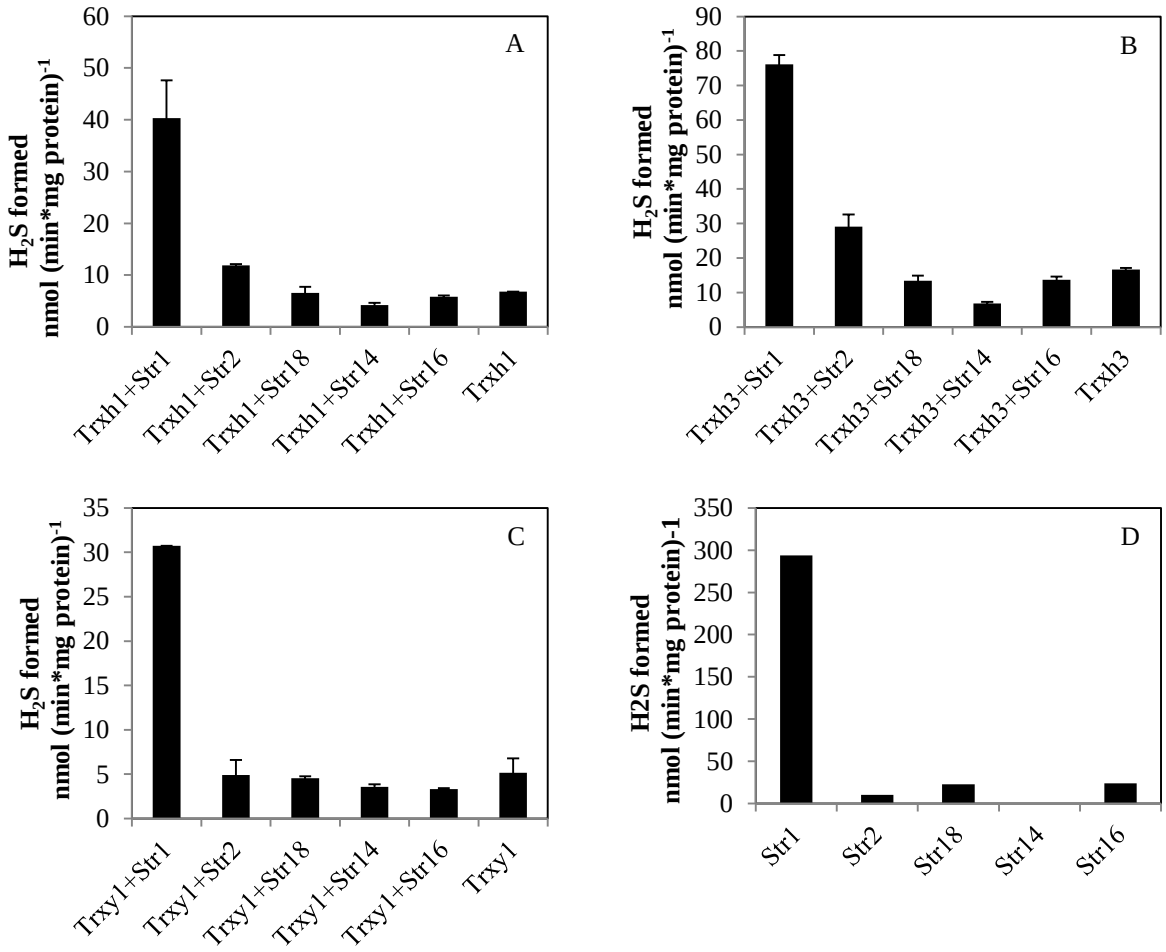


Figure S3. Combined enzyme assay using different Str and Trx and 50 µM 3-MP. A) H_2S test of Trxh1 with five different Str. B) H_2S test of Trxh3 with five different Str. C) H_2S test of Trxy1 with five different Str. D) H_2S test of four different Str without Trx.

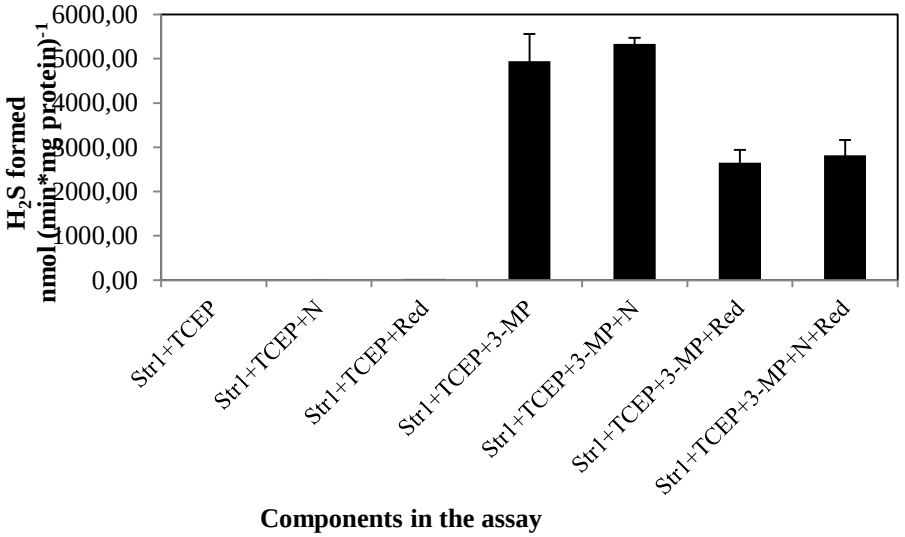


Figure S4. H_2S releasing assay of Str1 with each single component of the assay. Abbreviations: 3-MP, 3-mercaptopyruvate; N, NADPH; Red, thioredoxin reductase; Str, sulfurtransferase; TCEP, tris(2-carboxyethyl)phosphine. 3-MP concentration in the assay is 500 µM.

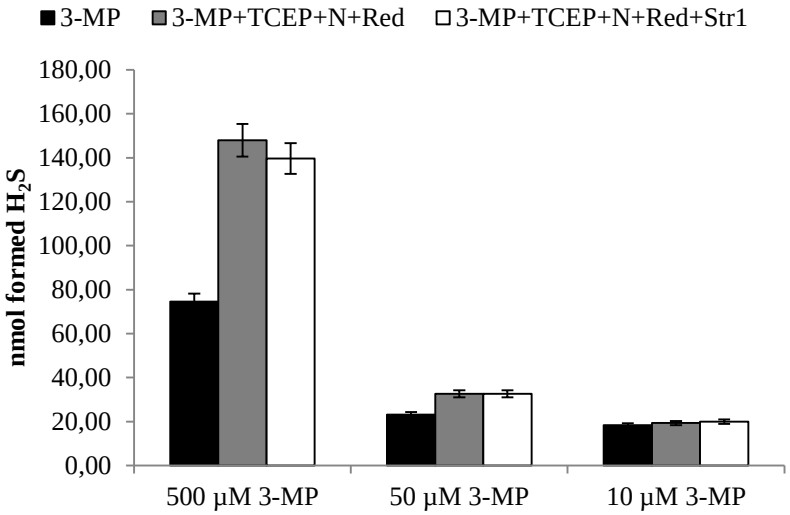


Figure S5. H_2S formed with different 3-MP concentrations (black bars), with different 3-MP concentration and the other components of the assay without Str (grey bars) and with different 3-MP concentration and the other components and 0.025 µM Str1 (white bars). Abbreviations: 3-MP, 3-mercaptopyruvate; N, NADPH; Red, thioredoxin reductase; Str, sulfurtransferase; TCEP, tris-(2-carboxyethyl)-phosphine.

CHAPTER 4

THE ROLE OF SULFURTRANSFERASES IN THE METABOLISM OF HIGHER PLANTS

Abstract

Sulfurtransferases (Str) comprise a group of enzymes widely distributed in all three phyla which catalyze *in vitro* the transfer of a sulfur atom from suitable sulfur donors to nucleophilic sulfur acceptors. Despite the presence of Str activities in many living organisms the physiological role of the members of this protein family is still not clear. Several functions were suggested, such as a role in cyanide detoxification in analogy to mammalian Str, the detoxification of free oxygen radicals in a thioredoxin reductase reaction, the mobilization and transport of reduced sulfur. To get some indications of the physiological role of Str in the metabolism of *Arabidopsis*, T-DNA insertion mutants of AtStr1, AtStr2, AtStr14 and AtStr15 were investigated. The effects of 1 mM thiosulfate (TS), 1 mM cysteine and 1 mM sulfite as well as different sulfates (125 µM and 3 mM) and phosphates (50 µM and 2.5 mM) on the germination were tested. TS and sulfite play a role in germination as shown by increased germination rates of mutants in the presence of these compounds. Additional Str enzyme activity and expression, total glutathione content and chlorophyll and carotenoid content were determined in comparison to wild-type plants but without any clear results. The findings indicate that Str play a role in sulfite metabolism.

INTRODUCTION

Sulfurtransferases (Str) are widely distributed in all three phyla. In *Arabidopsis thaliana* 20 putative members of this enzyme family exist which are divided into six groups and located in different compartments (Bauer et al., 2004; Peltier et al., 2004). 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 (TS) to cyanide, leading to the formation of sulfite and thiocyanate (Westley, 1973).

Although Str are widely distributed, the physiological role is not known to date. Based on the function in mammals it was proposed that Str play a role in cyanide detoxification but former experiments disprove this assumption (Chew, 1973; Meyer et al., 2003). In radish, tobacco and *Arabidopsis* dark-inducible, senescence-associated Str proteins encoded by *Rsdin1, Ntdin* and *AtSEN1 (AtStr15)* were identified and characterized (Oh et al., 1996; Chung et al., 1997; Shimada et al., 1998; Yang et al., 2003; Schenk et al., 2005). Of these the *Ntdin* gene product was shown to be involved in molybdenum cofactor (MoCo) biosynthesis (Yang et al., 2003). AtStr13 was found to be involved in auxin-signalling (Zhao et al., 2003) and also in the biosynthesis of the molybdenum cofactor synthesis (Matthies et al., 2004; Mendel, 2007). Mao et al (2011) showed that AtStr1 and AtStr2 play a role in embryo and seed development. In *Datura innoxia*, a cadmium-induced Str homologue (AtStr9) was identified, suggesting a role of Str in the reduction of heavy metal stress (Louie et al., 2003). Another important role played by Str is the maintenance of redox homeostasis by virtue of its ability to interact with thioredoxins (Trx) (Nandi et al., 2000; Ray et al., 2000; Nagahara et al., 2007; Sabelli et al., 2008).

Trx are small proteins (~12-14 kDa) catalyzing thiol-disulfide interchange and are involved in the regulation of the redox environment of the cell. In *Arabidopsis thaliana* more than 40 Trx and Trx-like genes have been reported in the whole sequenced genome (Meyer et al., 2005) and can be classified into six groups. Trx are located in different cell compartments (Gelhaye et al., 2005) and have all a conserved catalytic site (WCXXC). The major function of Trx is to reduce disulfide bridges in target proteins (Meyer et al., 2009).

In our former studies we showed an interaction of Str with Trx which indicates a function of Str in redox homeostasis (Henne et al. in preparation, see Chapter 3). With Northern blot analysis we try to find some indications of parallel expression of Str and Trx to prove the

BiFC results with another method. Additionally the expression studies should give some advice if one Str assumed the activity of another when this is knocked-out what we assumed. In this study we try to find some indication about the function of the Str in the metabolism of *Arabidopsis thaliana*. Based on studies done before with *Arabidopsis* wild-type (wtcol) plants (Bartels et al., 2007b) different supplemental substances for the medium were used. The stress conditions were chosen based on the microarray information in Genevestigator (www.genevestigator.com). The germination rates of four different Str T-DNA insertion mutants (*atstr1, atstr2, atstr14* and *atstr15*) on different MS media were determined. Str enzyme activity and expression were investigated in comparison to the wtcol plants. Additionally the total glutathione content and chlorophyll and carotenoid contents were measured. The increased germination rates of some mutants on MS medium with TS or sulfite revealed that these components play a role in plant development. The GSH and chlorophyll content did not give significant results and also the Str activity did not reveal any indications about the function of Str. The physiological role of Str seems to be more complicated than expected and more research needs to be done.

METHODS

Chemicals

All chemicals were obtained from Roth (Karlsruhe, Germany), AppliChem (Darmstadt, Germany) and Sigma (Taufkirchen, Germany) if not otherwise mentioned.

Plant growth and harvest

For the determination of the physiological role of *Arabidopsis* Str (AtStr) three SALK-mutants (*atstr2* SALK_067994; *atstr14* SALK_089057 and *atstr15* SALK_020571) and one SAIL-mutant (*atstr1* SAIL_69_D10) and *Arabidopsis thaliana* ecotype Colombia (wtcol) were ordered at the Nottingham Arabidopsis Stock Centre (NASC) and grown on MS medium (Murashige and Skoog, 1962) in a 12 h light/ 12 h dark cycle with about 100 µmol $m^{-2}s^{-1}$ light at 21°C.

In a second set of experiments seeds of *atstr2* and *atstr14* and wtcol were grown on MS medium (Murashige and Skoog, 1962). After three weeks the plants were transferred to soil in a propagation box - the mutant plants on the one side and the wtcol plants on the other side of the box. After three weeks growing in the greenhouse in a 12 h light/12 h dark rhythm the plants were put under stress conditions (Table 1). The stress conditions were chosen with the background of the information in Genevestigator (www.genevestigator.com) (Zimmermann et al., 2004; Hruz et al., 2008). Microarray data of the two Str were used for analyzing the gene expression. Only biotic and abiotic stress conditions leading to a more than two-fold up- or down-regulation of transcripts were selected for detailed analysis.

For tracking one life cycle seeds of the *atstr1, atstr2, atstr14* and *atstr15* and wtcol were sown on soil. After three weeks 24 plants were potted in pots of seven cm diameter and observed for seven weeks. After this period the numbers of siliques, the length of the stems and the section of the rosettes were determined. The plants were grown in a climate chamber under controlled conditions in a 8 h light/16 h dark rhythm with a quantum rate of 100 µmol $m^{-2}s^{-1}$ and 21°C.

Based on the experiments carried out with wtcol plants as described in Bartels et al. (2007), seeds of *atstr1, atstr2, atstr14, atstr15* and wtcol were grown on MS or ½ MS (Murashige and

Skoog, 1962) with supplemental substances: 1 mM cysteine, 1 mM TS, 1 mM sulfite, 50 µM sulfate (marked as –S), 3 mM sulfate (marked as +S), 125 µM phosphate (marked as –P), 2.5 mM phosphate (marked as +P). For each experiment 32 seeds were sown on eight plates, respectively. After six weeks the small plants were counted, including those that survived ones, to determine the germination rate (Equation [1]) and the survival rate (Equation [2]). The plants were harvested and frozen directly in liquid nitrogen. Each approach was repeated twice except the experiment with 1 mM TS.

[1] Germination rate [%]$= \frac{germinated\ plants}{32} * 100$

[2] Survival rate [%]$= \frac{survived\ plants}{32} * 100$

Table 1. Stress conditions. The mutants, and the detailed conditions are listed.

Mutant	**Stress situation**	**conditions**
atstr2	Control	growing in the greenhouse
	One hour 37°C	growing in a 37°C incubator for 1 h
	24 h 4°C	growing at 4°C for 24 h
	24 h dryness	growing 24 h without watering
	24 h darkness	growing in dark for 24 h
	150 mM NaCl	watering with 150 mM NaCl solution
	3 d dry and darkness	growing three days without watering and in darkness
atstr14	1 M NaCl 4 d	watering with 1M NaCl solution for 4 d
	0.5 M NaCl 4 d	watering with 0.5M NaCl solution for 4 d
	0.25 M NaCl 4 d	watering with 0.25M NaCl solution for 4 d
	0.15 M NaCl 4 d	watering with 0.15M NaCl solution for 4 d
	Control 4 d	watering with normal water for 4 d
	7 d darkness	growing the plants in darkness for 7 d
	7 d 4°C	growing the plants at 4°C
	7 d strong light	growing the plants at 350 µmol m^{-2} s^{-1}
	control 7 d	growing the plants at the greenhouse
	24 h at 37°C	growing the plants at 37°C for 24 h

DNA extraction

One leaf of a mutant plant was homogenized in 250 µl extraction buffer. Then 450 µl of the extraction buffer was added and the samples were incubated for 1 h at 65°C in a shaking incubator (Thermo scientific, Waltham, Massachusetts, USA). 700 µl chloroform/isoamylalcohol (24:1) were added and mixed thoroughly. After centrifugation for 10 min at 8.400*g* the upper phase was mixed carefully with 750 µl isopropanol in a new reaction tube. After centrifugation for 5 min at 8.400*g* the pellet was washed in 70% ethanol. The air dried pellet was dissolved in 50 µl Tris/EDTA buffer, pH 8.0, with 0.5 µg 100 μl^{-1} RNase at 65°C for 20 min.

PCR amplification

For detecting if the mutant plants were homozygous two different PCR reactions were necessary. One reaction with two gene-specific primers was performed to detect the wild-type allels and a second reaction with one gene-specific primer as well as T-DNA specific primer to detect the mutant allels (Østergaard and Yanofsky, 2004). Each PCR reaction tube contained 0.2 mM dNTPs, 0.4 mM of each primer (Eurofins MWG Operon, Ebersberg, Germany), 0.1 µl Dream *Taq* DNA polymerase (MBI Fermentas, St. Leon-Roth, Germany) and about 0.5 µg template DNA in a final volume of 20 µl. Before starting the PCR cycle, the DNA was denatured for 180 s at 94°C followed by 28 PCR cycles conducted for 60 s at 94°C, 60 s at the different annealing temperatures required, and 60 s at 72°C. The process was finished with an elongation phase of 600 s at 72°C. The PCR product was loaded on a 1% agarose gel and stained with midori green (Biozym, Hessisch Oldendorf, Germany). The primers and the conditions of the PCR are listed in Table 2.

The PCR for the digoxygenin (DIG) labeled probe was done with the PCR DIG probe synthesis Kit (Roche, Mannheim, Germany). As template the respective PCR product was used. Fifty µl comprised 5 µl template (PCR product), 5 µl PCR buffer, 5 µl PCR DIG-mix, 1 µl of each primer and 0.75 µl polymerase mix filled up with water to 50 µl. The DIG PCR was verified on a 1% agarose gel. The DIG PCR product was dissolved in 25 ml of prehybridisation solution (7% SDS, 40% formamide, 5x SSC, 2% blocking-solution (from the DIG wash and block kit), 50 mM sodiumphosphate pH 7.0, 0.1% N-laurysarcosine).

Table 2. List of primers. The gene name, the sequence of the primer with the lab intern number and the annealing temperature are listed.

Gene name	Primer pairs	T_A
AtStr1	P9 CGCTGCAGTCATGAAGATTCA	55°C
	P12 CAGGTACCGCTTCTACTGGAGTT	
AtStr 2	P32 CGGATCCGCTTCTTCTGGATCTGA	55°C
	P33 CCTGCAGTGAAGAAGAACCCACT	
AtStr14	P118 CGGATCCGCTTCACTTACTTCAAT	52°C
	P119 CGGTCGACAGTCTTCTTCAATTGT	
AtStr15	P110 CGGATCCGAAACCACTGCTTTTAAC	55°C
	P111 CGGATCCGAAACCACTGCTTTTAAC	
Trxf1	P688 TCTAGACCTCTTTCTCTCCGT	54°C
	P689 CTCGAGTCCGGAAGCAGCAGA	
Trxh1	P664 TCTAGAGCTTCGGAAGAAGGA	54°C
	P665 CTCGAGTTAAGCCAAGTGTTT	
Trxo1	P656 TCTAGAAAGGGAAATTGGTCG	52°C
	P657 CTCGAGTCACTTGTAGAGCTG	
LB *npt*	P547 TGGTTCACGTAGTGGGCCATCG	55°C
	P548 TCAAACAGGATTTTCGCCTGCT	52°C
LB *bar*	P629	55°C
	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC	

npt neomycin phodphotransferase; *bar* bialaphos resistance gene

RNA extraction and Northern Blots

Total RNA was extracted essentially as described in Sokolovsky et al. (1990) RNA samples of 15 µg were separated in 1% denaturing agarose formaldehyde gels. Equal loading was controlled by staining the gels with ethidium bromide. After RNA-transfer onto nylon membranes and hybridization with a DIG-labeled probe, chemiluminescent detection method with CDP-Star (Roche) or colorimetric detection with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) was applied.

Determination of the sulfurtransferase activity

The plant material was ground with 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 1:6 (100 mg plant material plus 500 µl buffer). After centrifugation the protein content of the supernatant was used. The determination of Str enzyme activity using 3-mercaptopyruvate (3-MP) or thiosulfate (TS) as sulfur donor was performed as described in Papenbrock and Schmidt (2000). For the plants grown on MS or ½ MS medium with different supplemented chemicals the concentration of the supernatant was adjust to 0.25 µg μl^{-1}. The total amount of protein in the assay was 25 µg. Protein concentrations were determined according to Bradford (1979) using bovine serum albumin as standard.

Chlorate sensitivity test

To investigate a possible role of AtStr15 in molybdenum cofactor biosynthesis a chlorate sensitivity test was conducted according to Yang et al. (2003). Wtcol and *atstr1, atstr2, atstr14* and *atstr15* were germinated on MS medium or 1/2 strength MS medium in a climatic chamber at 12 h light/12 h dark rhythm. After 4 weeks of growth, plants were transferred to fresh MS medium or 1/2 strength MS medium or double strength MS with or without addition of 5 mM potassium chlorate. The plant growth was visually controlled for 30 days.

Chlorophyll and carotenoid determination

On 50-100 mg fine powdered plant material 400 µl ice-cold 80% acetone was pipetted and incubated on ice for 10 min. From time to time the samples were vortexed. After centrifugation for 5 min at 14.000*g* the supernatant was put in a new reaction tube and hold on ice. Three times 200 µl of the 80% acetone were added to the pellet and 5 min centrifugation at 14.000*g* followed every time. The supernatants were combined with the others. For the spectrophotometrical determination of the chlorophyll and the carotenoid contents the sample was diluted 1:20 with 80% acetone and measured at the wavelengths 663.2 nm, 646.8 nm, 470 nm and 750 nm. The calculation was done as described in Wellburn (1994)

Determination of the reduced total glutathione content

The total glutathione content was measured as described in Sari-Gorla et al. (1993). 60 mg plant material was homogenized in 5% trichloroacetic acid (TCA) and centrifuged for 15 min at 12.000*g*. The supernatant was brought to pH 4-5 with 1 N NaOH. 100 µl sample was mixed with 100 µl 5.5-dithiobis-2-nitrobenzoic acid (DTNB 59 mg/100 ml Tris/HCl buffer 0.1 M, pH 7.0). The absorbance of the mixture was read in a microplate reader (Synergy Mx, BioTek, Bad Friedrichshall, Germany) at 412 nm. The glutathione concentration was calculated from the standard curve using 2-20 µg ml^{-1} of reduced glutathione.

Miscellaneous

Statistical analysis, standard derivation and Student's t-test was done with Microsoft Excel 2007.

RESULTS

Development of the plants

Before starting the experiments the plants were tested if the mutation was homozygous. Two PCR reactions were done and the PCR products were loaded on a 1% agarose gel and stained with midori green to check the size of the fragments. In Figure 1 the location of the insertion and the size of the fragments are shown. If the plants were homozygous no bands with the gene-specific primer and one band with the T-DNA-specific primer was seen in the gel. Two bands were seen, one with gene-specific primer and one with T-DNA-specific primer, when the plants were heterozygous and just one band with the gene-specific primer were seen in the gel if the plants were wtcol-like. In Table 3 the total numbers of screened plants are listed. The plants of *atstr2, atstr14* and *atstr15* were screened in former works (Bartels, 2006) and so homozygous seeds existed. The PCR reactions done for these mutants were to verify the mutation. For *atstr1* a lot of PCR reactions had to be done before homozygous seeds could be harvest. All T-DNA insertions were located in an exon except SALK_020571 (*atstr15*), there was the insertion in an intron (Fig. 1A-D).

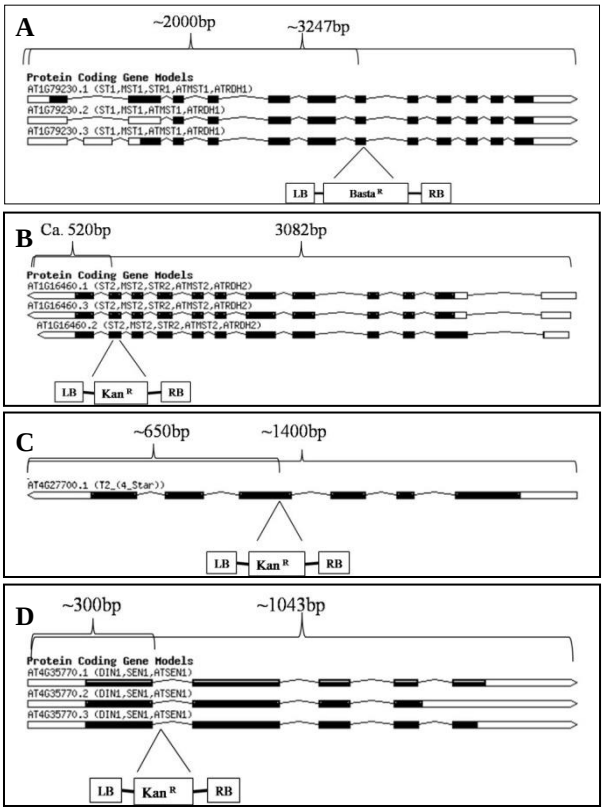


Figure 1. Insert location of the T-DNA in the different mutants. The models were done with the information of TAIR http://gbrowse.arabidopsis.org **(A)** Insert location of SAIL_69_D10 (*atstr1*)**; (B)** Insert location of SALK_067994 (*atstr2*)**; (C)** Insert location of SALK_089057 (*atstr14*)**; (D)** Insert location of SALK_020571 (*atstr15*). LB is the left border primer and RB is the right border primer.

Table 3. Results of the PCR screening of the mutants. The mutant, the total number of screened plants and the zygosity are listed.

Mutant	Total screened	Homozygous	Heterozygous	wt-like
SAIL_69_D10 (*atstr1*)	224	11	200	13
SALK_067994 (*atstr2*)	15	14	0	1
SALK_089057 (*atstr14*)	35	35	0	0
SALK_020571 (*atstr15*)	22	22	0	0

For checking if the mutation has an effect on the development of the plants, seeds of *atstr1, atstr2, atstr14, atstr15* mutants and wtcol were sown on soil and observed over ten weeks. For every mutant and wtcol 24 plants were observed. After this period the length of the stem, the number of siliques, the number of leaves and the size of the rosettes were determined (Fig. 2). The general observations of the mutant and wtcol plants showed that the phenotypes of the mutant plants did not differ from the phenotype of the wtcol plants. There was a slightly delayed development observable. The wtcol plants started flowering after seven weeks while the mutants started flowering one week later. After five weeks the size of the rosettes of the wtcol plants was on average 6 cm and of the mutant plants 4.5 cm. After ten weeks the size of the rosettes at the wtcol plants was on average 14 cm this was also the case for *atstr1* and *atstr2*. Just *atstr14* and *atstr15* showed a lower size, in average 12 cm for both (Fig. 2D). The results of the siliques showed the clearest result. The wtcol plants produced more than twice as many as the mutant plants. The comparison showed that all mutants differ highly significantly from the wtcol. The smallest amount of siliques showed *atstr2* which differed highly significantly from *atstr14* and significantly from *atstr15.* The siliques amount in *atstr1* was nearly the same as in *atstr2* (Fig. 2B). A similar result was also obtained by comparing the stem length of the mutants with wtcol. The wtcol stem was taller than the stems of the mutant plants (Fig. 2A). When comparing the number of leaves it was recognizable that there were no differences between *atstr14* and wtcol but *atstr1, atstr2* and *atstr15* showed higher amount of leaves in comparison to wtcol plants.

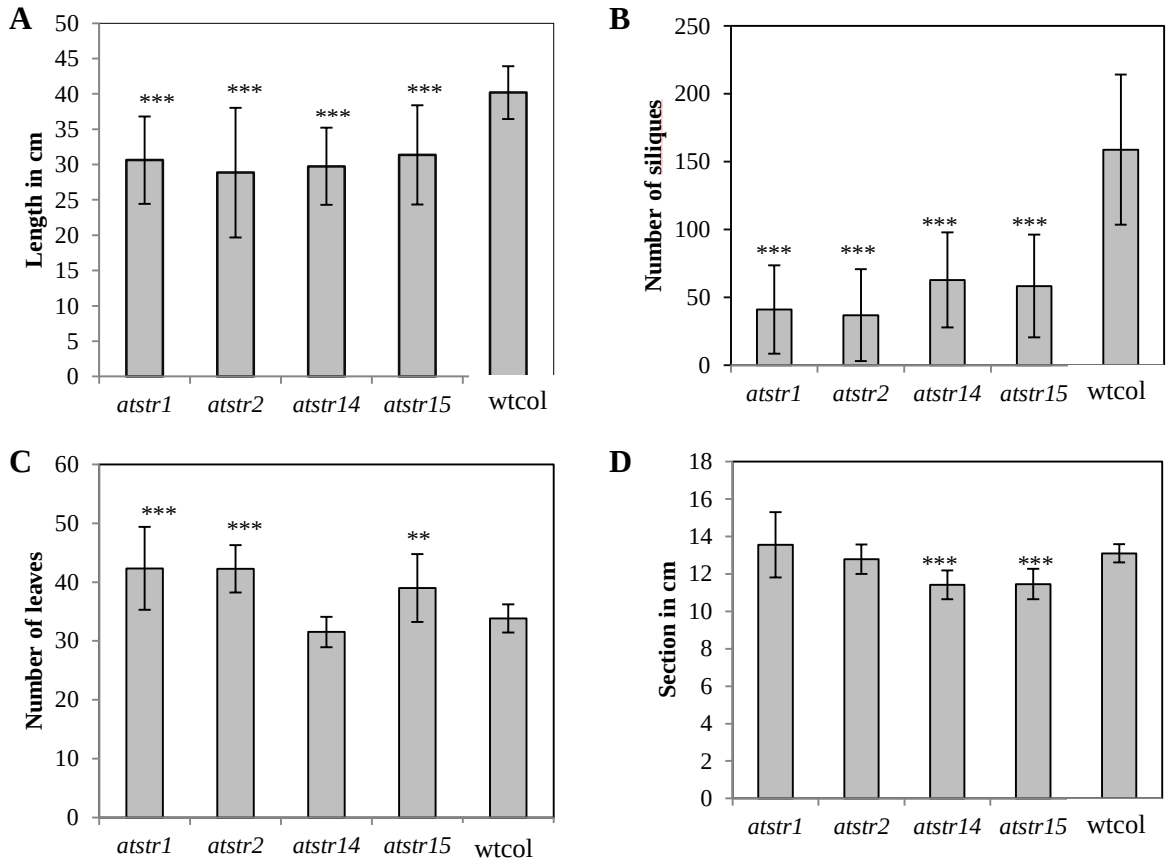


Figure 2. Results of the life cycle experiment. After ten weeks growing on soil under controlled condition (12h/12h light/dark 100 µmol $s^{-1}m^{-2}$ and 22°C) the leaves and the siliques were counted and the length of the stem and the section of rosettes were determined. (A) The average stem length (B) numbers of the silique (C) numbers of the leaves (D) section of the rosette. For each mutant and wild-type (wtcol) 24 plants were used. The experiment was done once. The black bars are the standard deviation and asterisks marked the significant differences between mutants and wtcol with the following classification: *** p<0.001; ** p<0.01 (Student's t-test calculated with Excel).

Looking at the results of the germination rate (Fig. 3A) it is noticeable that the germination rate of wtcol was only about 50% but it should be 100%. This low germination rate can also be observed on the control medium (MS without any substances). A reason for this could be the fact that the seeds were too old and did not have a good vitality anymore. A significant increase in the germination rate was observed with the medium containing 1 mM sulfite. Here, the germination rate was almost 100%. On all media a germination rate of 30-40% was noticed except on the medium with 1 mM TS. An increased germination rate up to 90% was observed here. A similarly low germination rate of 30-40% on the different media showed *atstr14*. Also here is an exception on the medium with 1 mM TS. The germination rate increased to 65%. The seeds of *atstr2* showed a quite high germination rate on MS (70%), MS+Cys (80%), MS+TS (100%) and MS+sulfite (100%). On the other used media, MS+S,

MS-S, MS+P and MS-P the germination rates were about 30%. These germination rates were observable for all mutants and wtcol on this media. Beside *atstr2* also *atstr15* showed a high germination rate of 100% on MS, MS+Cys, MS+TS and MS+sulfite. Taking a closer look on the germination rates and the survival rates of the plants on MS+sulfite it was noticeable that the germination rates of *atsr2, atstr15* and wtcol was quite high, but only 15% and 25% of the plants of *atstr15* and wtcol survived, respectively, while 75% of *atstr2* were alive after six weeks (Fig. 3A and B).

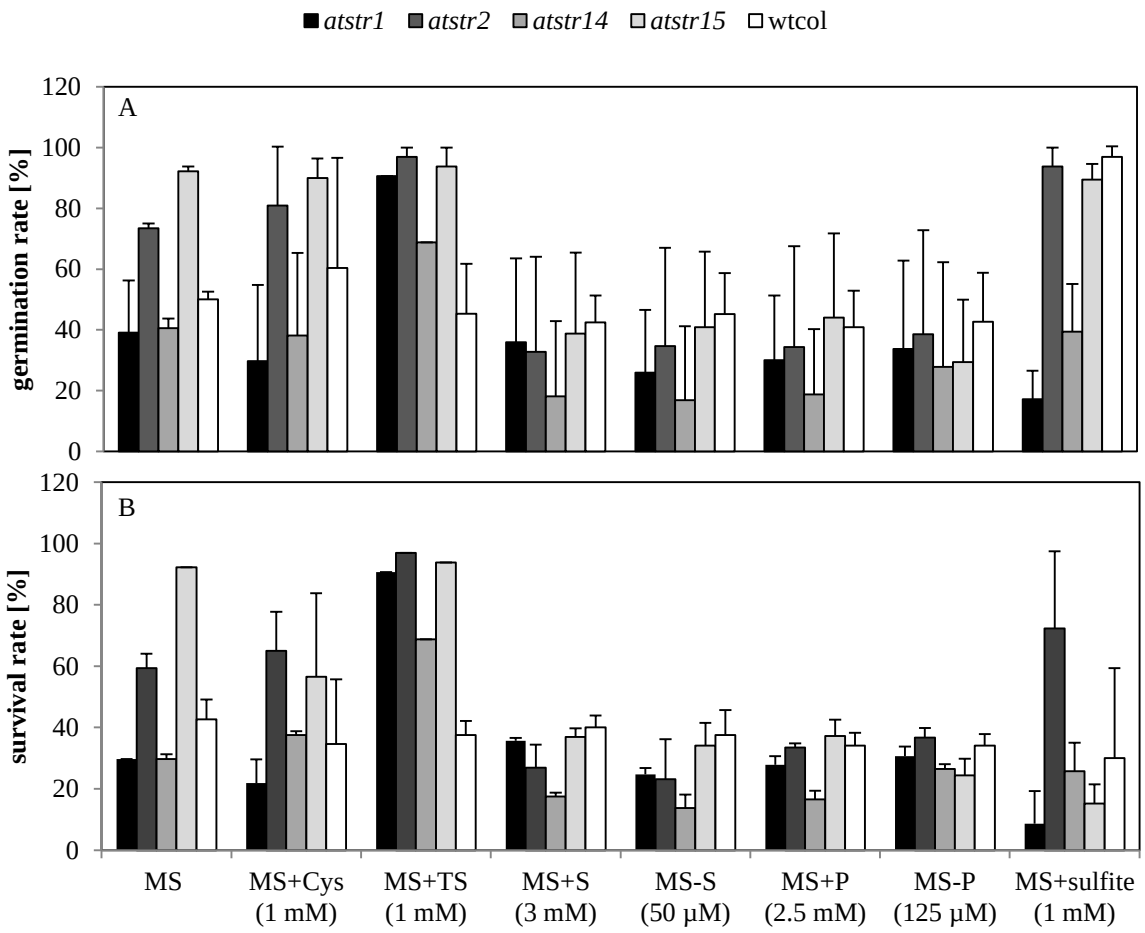


Figure 3. Germination rate and survival rate of plants on MS medium supplemented with different substances. Seeds of *atstr1, atstr2, atstr14, atstr15* and wtcol were grown on MS medium with supplemented substances. For every approach eight plates with 32 seeds were used. (A)The germinated plants were counted after six weeks. (B) The survived plants were counted after six weeks. The experiments were done twice. The percentage of germinated seeds and survived plants and the standard deviation are given in the diagram.

For the stress conditions wtcol plants and *atstr2* or *atstr14* were put together in a box on soil. This ensures that wtcol and mutants were exposed to the same conditions. Both, before and after the application of stress, the phenotype of the mutants did not differ from the phenotype of wtcol (Fig. 4). The plants were exposed to the stress conditions over seven days except for the salt stress experiment. This was stopped after four days because the plants were severely damaged after this time period and would die if the experiment took more days. The leaves of the plants watered with 1 M NaCl were severely damaged or had necrotic spots and did not have turgor anymore (Fig. 4A). The plants which were watered with 0.5 M NaCl showed some necrotic leaves. Some of the older leaves were violet-colored by stress-induced anthocyanins but the turgor of the younger leaves seemed to be not much influenced (Fig. 4B). The plants from the experiment with 150 mM NaCl and 250 mM NaCl had the same appearance (Fig. 4D and 4C). Some of the older leaves showed a violet coloration and necrotic spots. The younger leaves looked healthy and phenotypically undamaged. The cold stressed and light stressed plants had both violet colored leaves. The plants exposed to strong light also started flowering (Fig. 4E) while the cold stressed plants did not produce any stems (Fig. 4F).

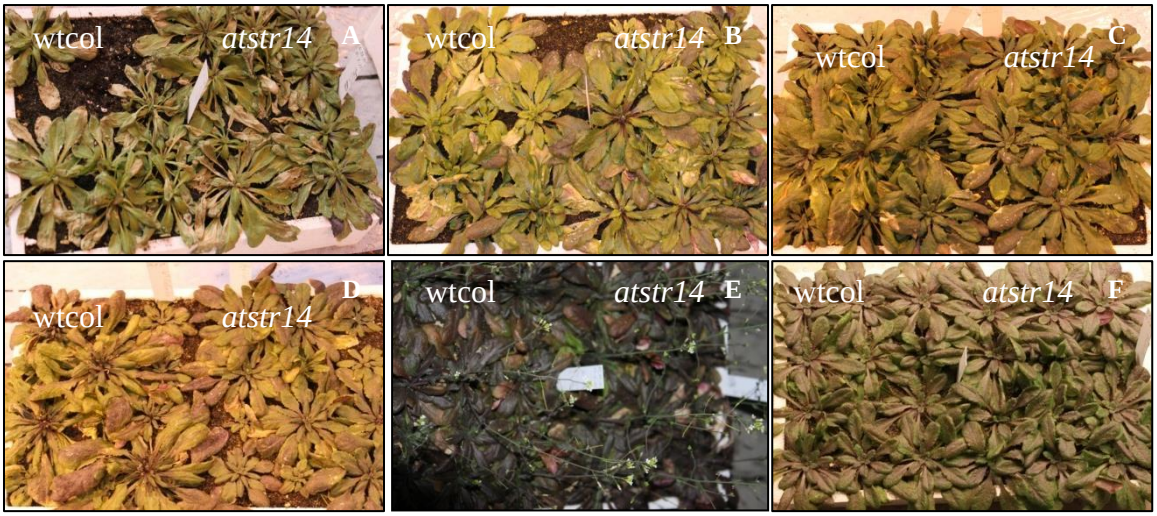


Figure 4. Stressed plants of *atstr14* and wtcol. Plants of *atstr14* and wtcol together in one box exposed to different stress conditions for 4 or 7 days. A) 1 M NaCl for 4 d; B) 0.5 M NaCl for 4 d; C) 0.25 M NaCl for 4 d; D) 0.15 M NaCl for 4 d; E) strong-light for 7 d; F) 4°C for 7 d

Expression studies

The RNA of *atstr2* and *atstr14* was extracted and loaded on a gel. After transfer on a membrane it was hybridized with a DIG-labeled probe and detected with CDP-Star or NBT/BCIP. The membranes were hybridized with different Str probes to see if there were any indications that another Str take over the function of the knocked-out Str. The hybridization with different Trx probes should give some indications of their parallel expression based on the results of Chapter 3. The expression study of *atstr2* showed that the gene for AtStr2 was completely down-regulated. Figure 5B showed the membrane which was detected with NBT/BCIP. It was visible that there were just bands at the wtcol samples. Because of the poor recognizability of the bands, differences between the single approaches were almost undetectable. The expression of *AtStr1* (Fig. 5A) was slightly increased for the samples treated with 150 mM NaCl (sample 11 and 12). The bands were somewhat stronger than the bands of the control group (sample 1 und 2). Furthermore there was a decreased expression of the samples treated three days in darkness and dryness (sample 13 and 14). If one compared these samples with the other membranes it revealed that all tested genes were not expressed under dark and drought stress. The bands of the samples treated with 150 mM NaCl (sample 11 and 12) on the membrane hybridized with the probe *AtStr14* were also stronger than the control ones (Fig. 5C). Here, it was also visible that the band of the mutant sample was twice as strong as the wtcol sample. There was also a stronger band at the mutant sample treated 24 h with drought (sample 8) in comparison to the corresponding wtcol sample (sample 7). The hybridization of the membrane with probe *Trxf1* (Fig. 5D) showed a strong expression in the control samples (sample 1 and 2). The samples treated for one hour at 37°C (sample 3 and 4) showed a decreased expression in which the band of the mutant was weaker in comparison to the wtcol. In comparison to the control samples, the samples of the treatment 150 mM NaCl showed a weaker expression while all other genes showed a higher expression of these two samples. The bands at the membrane hybridized with probe *Trxo1* (Fig. 5E) were very weak but it was recognizable that the wtcol control band was detectable while the mutant band was not. Here, it was also discernable that the bands of the samples treated with 150 mM NaCl (sample 11 and 12) were stronger than the control ones in which the sample 11 was slightly stronger. On the membrane, which was hybridized with *Trxh1* it was really difficult to recognize bands (Fig. 5F). It was not possible to make a conclusion about the expression pattern. The membrane with the RNA of mutant *atstr14* which was hybridized with *Trxh1* also shows no bands (data not shown). Based on these results, the probes were tested for their

functionality. It was found that all the probes hybridized only to the respective plasmid DNA. Only the probe *Trxy1* showed cross-hybridization and was therefore not used in the expression study (data not shown).

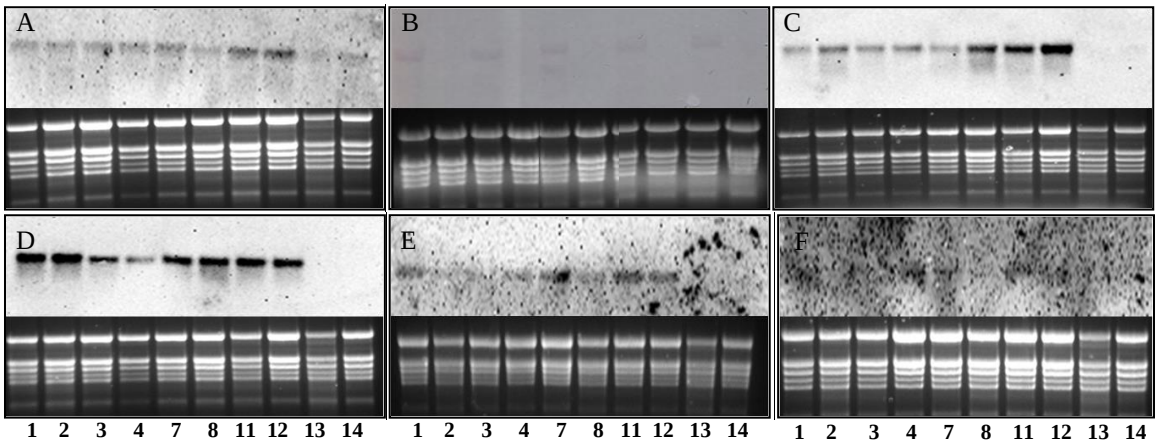


Figure 5. Northern Blot Analysis of *atstr2*. Plants were grown on soil for six weeks and were then exposed to different stress conditions. Fifteen µg of total RNA extracted from *atstr2* and wtcol were hybridized with probe A) *AtStr1* B) *AtStr2* C) *AtStr14* D) *Trxf1* E) *Trxo1* and F) *Trxh1* here 20µg of the RNA were loaded. Lane 1 (wtcol), 2 (mutant) control; 3 (wtcol), 4 (mutant) one hour at 37°C; 7 (wtcol), 8 (mutant) 24 h dryness; 11 (wtcol), 12 (mutant) 150 mM NaCl; 13 (wtcol), 14 (mutant) three days darkness and dryness.

In the expression analysis of *atstr14* it was also visible that the gene for AtStr14 is switched off because there were just bands in the wtcol samples (Fig. 6B). A general problem of the RNA samples from *atstr14* was that an equal loading on a gel was not possible. There were many attempts, but it never worked in a satisfying way. The best results were used for the transfer to a membrane and hybridized with different probes. In the hybridization with probe *AtStr2* it was noticeable that the bands of samples which were treated 24 h at 37°C (sample 1 and 2) were much stronger compared to the control samples (sample 11 and 12). The samples 3 and 4 (1 M NaCl) did not show any bands. All other samples showed the same weakness of the bands. The minimal differences that were recognizable came from the unequal loading (Fig. 6A). In the 7 days control plants *Trxf1* showed a five times higher expression in the mutant plants than in wtcol plant (Fig. 6C, samples 19 and 20) whereas the 4 days control plants did not show any differences between mutant and wtcol sample (Fig. 6C, samples 11 and 12). The bands of these samples were also much stronger than the other control samples. Comparison of the expression of the salt stressed plants with control showed the same expression intensity of control and samples treated with 0.25 M NaCl. The bands of the

samples treated with 0.5 M NaCl showed the half the intensity as the control. The samples treated with 0.15 M NaCl showed bands with about 25% intensity of control whereas the samples treated with 1 M NaCl showed no expression. Differences between wtcol and mutant were not visible. Also no expression was detected in the dark stressed samples (13 and 14). The bands of the cold stressed plants and light stressed plants revealed a weaker intensity in the mutant and stronger intensity on wtcol in comparison to the control. The experiment was done once and has to be repeated to verify the results.

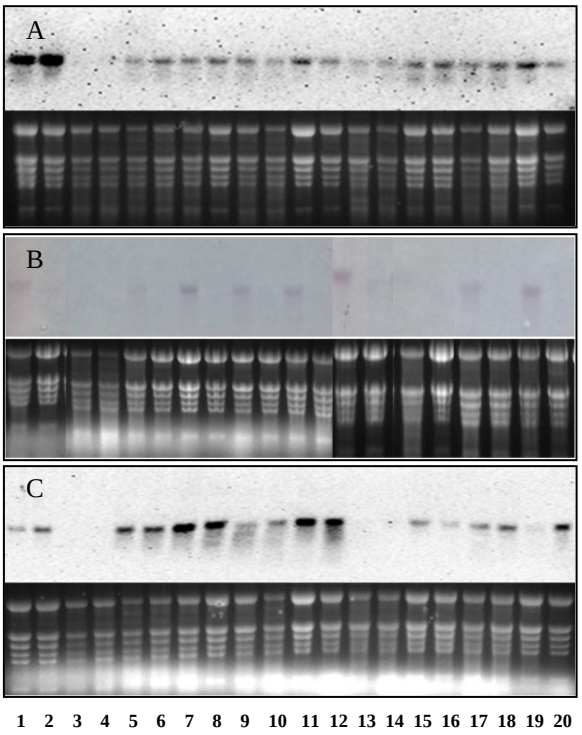


Figure 6. Northern Blot analysis of *atstr14*. Plants were grown for six weeks on soil in the greenhouse and were then exposed to stress conditions. Fifteen µg of total RNA extracted from *atstr14* and wtcol were hybridized with probe A) *AtStr2* B) *AtStr14* and C). *Trxf1* Lane 1 (wtcol), 2 (mutant) 24h at 37°C; 3 (wtcol), 4 (mutant) 4 days 1 M NaCl; 5 (wtcol) 6 (mutant) 4 days 0.5 M NaCl; 7 (wtcol), 8 (mutant) 4 days 0.25 M NaCl; 9 (wtcol), 10 (mutant) 0.15 M NaCl; 11 (wtcol), 12 (mutant) control 4 days; 13(wtcol), 14 (mutant) 7 days darkness; 15 (wtcol), 16 (mutant) 7 days at 4°C; 17 (wtcol), 18 (mutant) 7 days strong light; 19 (wtcol), 20 (mutant) control 7 days.

Biochemical analysis of the mutants

The Str activity was measured in total soluble protein extracts from the mutant plants and wtcol grown on MS or ½ MS medium. The results are shown in Figure 7. The sulfur donor for this measurement was 3-MP and cyanide was the acceptor. On ½ MS medium *atstr1* and *atstr15* had the same Str activity of about 80 nmol min^{-1} mg^{-1} which was significantly lower

than in wtcol. The wtcol, *atstr2* and *atstr14* had the same Str activity of 120 nmol min^{-1} mg^{-1}. The activity of *atstr2*, *atstr15* and wtcol on MS medium was about 110 nmol min^{-1} mg^{-1} while the activity of *atstr1* was significant lower (70 nmol min^{-1} mg^{-1}). The mutant of AtStr14 showed a higher activity of 150 nmol min^{-1} mg^{-1} in comparison to wtcol.

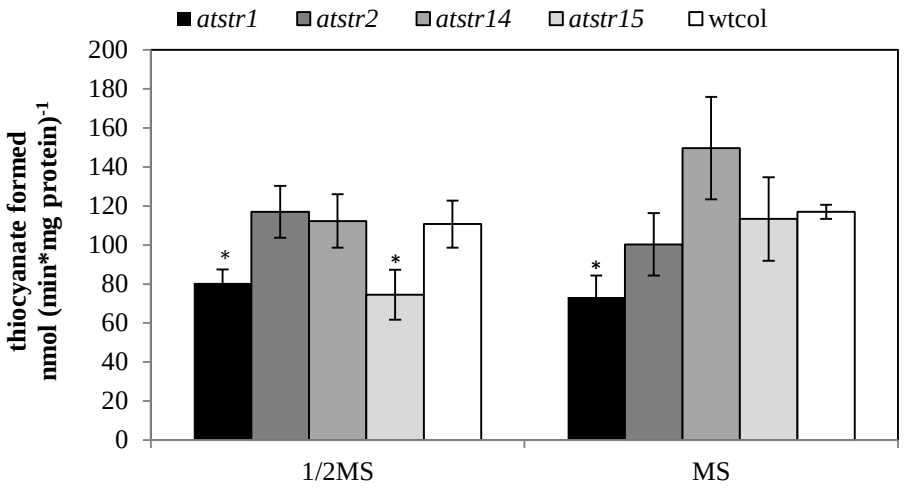


Figure 7. Sulfurtransferase (Str) enzyme activity of mutants grown on ½ MS and MS medium. Plants were grown for six weeks on MS or ½ MS plates and Str activity was determined in crude protein extracts of the soluble protein fraction from wtcol, *atstr1*, *atstr2*, *atstr14* and *atstr15* using 3-MP as sulfur donor. Three independent experiments were done. Asterisks marks the significant differences between wtcol and mutants (*$p<0.05$)

The Str activity was also measured with the total protein extract of *atstr14* and wtcol exposed to different stress conditions. In Figure 8 the results of the salt stressed plants are given. There were no differences between wtcol and *atstr14*, just *atstr14* treated with 0.5 M NaCl showed a slightly higher activity (203 nmol min^{-1} mg^{-1}) than wtcol (173 nmol min^{-1} mg^{-1}. The samples treated with 0.15 M NaCl showed a higher activity in general of about 200 nmol min^{-1} mg^{-1} while all other samples showed an activity of about 150 nmol min^{-1} mg^{-1}.

The results of the measurement of the plants stressed with darkness, cold and light were shown in Figure 9. Only differences between wtcol and *atstr14* were observed in the control group and in the light stressed plants. In the control group the Str activity of *atstr14* was lower (99 nmol min^{-1} mg^{-1}) than in wtcol (161 nmol min^{-1} mg^{-1}) while in the light stressed plants the activity was higher in *atstr14* (196 nmol min^{-1} mg^{-1}) than in wtcol (122 nmol min^{-1} mg^{-1}). The

Str activity in plants stressed with cold or darkness were at the same level in wtcol and *atstr14*. A statistical analysis was not possible because the measurement was done once. From the stress experiments done with *atstr2* no Str activity could be measured because of missing plant material.

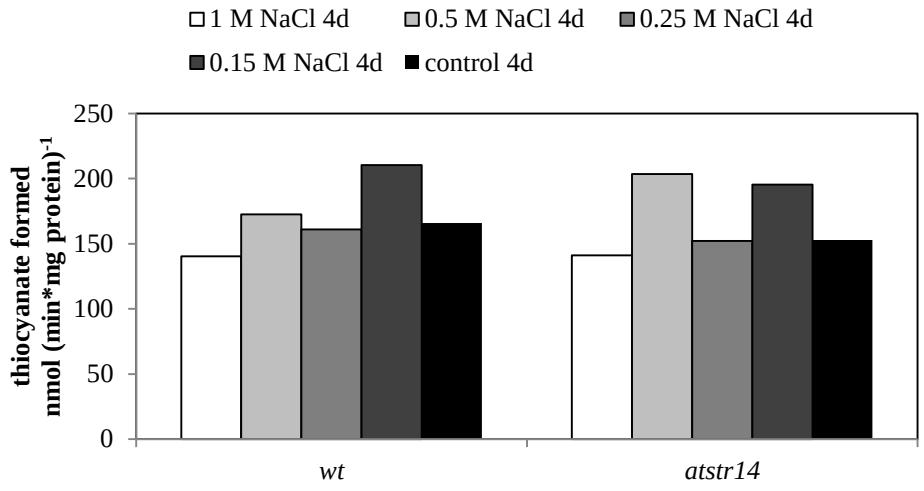


Figure 8. Sulfurtransferase activity of *atstr14* and wtcol under stress conditions for 4 days. Mutant plants of AtStr14 and wtcol grown on soil for six weeks in the greenhouse at 22°C and 120 µmol min^{-1} s^{-2}. After four days watering the plants with different NaCl-solutions the Str activity was determined in total protein extracts using 3-MP as sulfur donor. The activities were measured once.

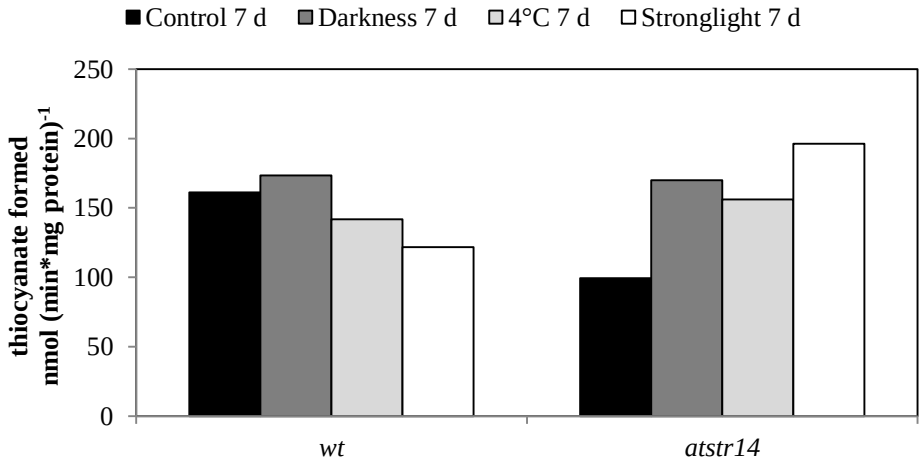


Figure 9. Sulfurtransferase activity of *atstr14* and wtcol under stress condition for 7 days. Plants of *atstr14* and wtcol grown on soil for six weeks in the greenhouse at 22°C and 120 µmol min^{-1} s^{-2} and then exposed to different stress conditions for seven days. Str activity was determined in crude protein extracts using 3-MP as sulfur donor. The activities were measured once.

For the determination of reduced glutathione, a plant extract with TCA (5%) was prepared and measured at 412 nm in a microplate reader (Biotek) after adding DTNB. Plant material of plants grown on MS or ½ MS medium with supplemental substances was used. Because the results on both media types were almost the same just the GSH contents of plants grown on MS medium shown in Figure 10. On MS medium (control) all mutants and wtcol had almost the same GSH content of about 50 µg g^{-1} FW. For the other conditions it was difficult to give a clear conclusion about the results of the GSH measurement. The highest amount of reduced glutathione was measured in plants grown on MS medium with 1 mM sulfite (between 60 and 75 µg g^{-1} FW). In the mutant plants grown on MS-S the lowest amount of GSH was determined (between 10 and 20 µg g^{-1} FW) while in wtcol an amount of 50 µg g^{-1} FW was determined. On MS+P and MS-P the GSH content in the plants was like in the control group (50 µg g^{-1} FW). Only *atstr14* on MS-P showed a higher amount of reduced glutathione (72 µg g^{-1} FW). On MS medium supplemented with TS or Cys the measured GSH content was lower than the control group. For a*tstr14*, *atstr15* and wtcol an amount of about 50 µg g^{-1} FW was measured in plants grown on MS+S while *atstr1* and *atstr2* had a smaller amount.

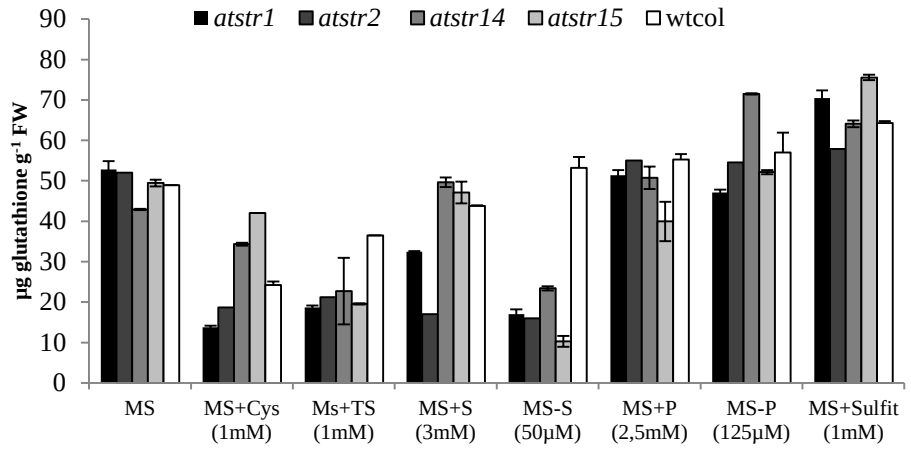


Figure 10. **Total glutathione content.** Seeds of the mutant and wtcol were sown on MS plates with supplemental substances. After six weeks the plants were harvested. With trichloroacetic acid (TCA) (5%) the total glutathione content get extracted and measured after addition of 5,5-dithiobis-2-nitrobenzoic acid (DTNB) at 412 nm.

Table 4 shows the chlorophyll a and b contents and the carotenoid content measured with spectrophotometer. Comparing the chlorophyll (chl) a contents *atstr1* had the lowest (384.58 µg g^{-1} FW) and *atstr15* shows the highest amount (789.53 µg g^{-1} FW). The chl a content of wtcol was 593.90 µg g^{-1} FW. Nearly the same amount was measured in *atstr2* (537.10 µg g^{-1} FW) and *atstr14* had a chl a content of 478.65 µg g^{-1}FW. The same tendency were seen in the carotenoid measurement. The ratio between chl a and chl b gave another result. Here *atstr1* had the highest ratio of 3.52. The general ratio in plants of 3 just had *atstr2* (3.05) and wtcol (3.08) while *atstr14* and *atstr15* had a higher ratio of 3.12 and 3.17, respectively. These measurements were just done once and have to be repeated to verify these results.

Table 4. Chlorophyll and carotenoid contents. The chlorophyll a content, the chlorophyll b content, the ratio of chlorophyll a to chlorophyll b and the carotenoid content in *atstr1, atstr2, atstr14, atstr15* and wtcol. The chlorophyll and carotenoid contents were determined spectrophotometrically once and are given in µg g^{-1} FW.

	atstr 1	***atstr 2***	***atstr 14***	***atstr 15***	**wtcol**
chlorophyll a	384.58	537.10	478.65	789.53	593.90
chlorophyll b	109.27	176.15	153.58	249.21	192.66
chlorophyll a/b	3.52	3.05	3.12	3.17	3.08
carotenoid	92.85	134.81	120.51	201.95	157.60

Chlorate sensitivity test

In the chlorate sensitivity test a possible involvement of AtStr15 and the other mutants of AtStr1, AtStr2 and AtStr14 in MoCo biosynthesis was investigated according to Yang et al., (2003). Chlorate is a toxic substrate analogue of nitrate reductase. Plants deficient in MoCo biosynthesis will not be able to produce functional nitrate reductase or other MoCo-proteins. Plants without functional MoCo factor and consequently without functional nitrate reductase will not be able to use chlorate, while wtcol plants metabolizing the toxic substrate analogue will suffer intoxication. For the chlorate test seeds of *atstr1, atstr2, atstr14, atstr15* and wtcol were sown on ½ MS or MS medium. After four weeks, the plants were placed in fresh medium or medium containing 5 mM $KClO_3$. Plant growth was observed for 30 days. After 30 days it was clearly visible that the plants grown on ½ MS with 5 mM $KClO_3$ all died. No differences between mutants and wtcol were observed (Fig. 11). The plants on ½ MS without

chlorate looked developed normally. On MS and MS with 5 mM $KClO_3$ all plants survived and no differences between mutants and wtcol were observed either.

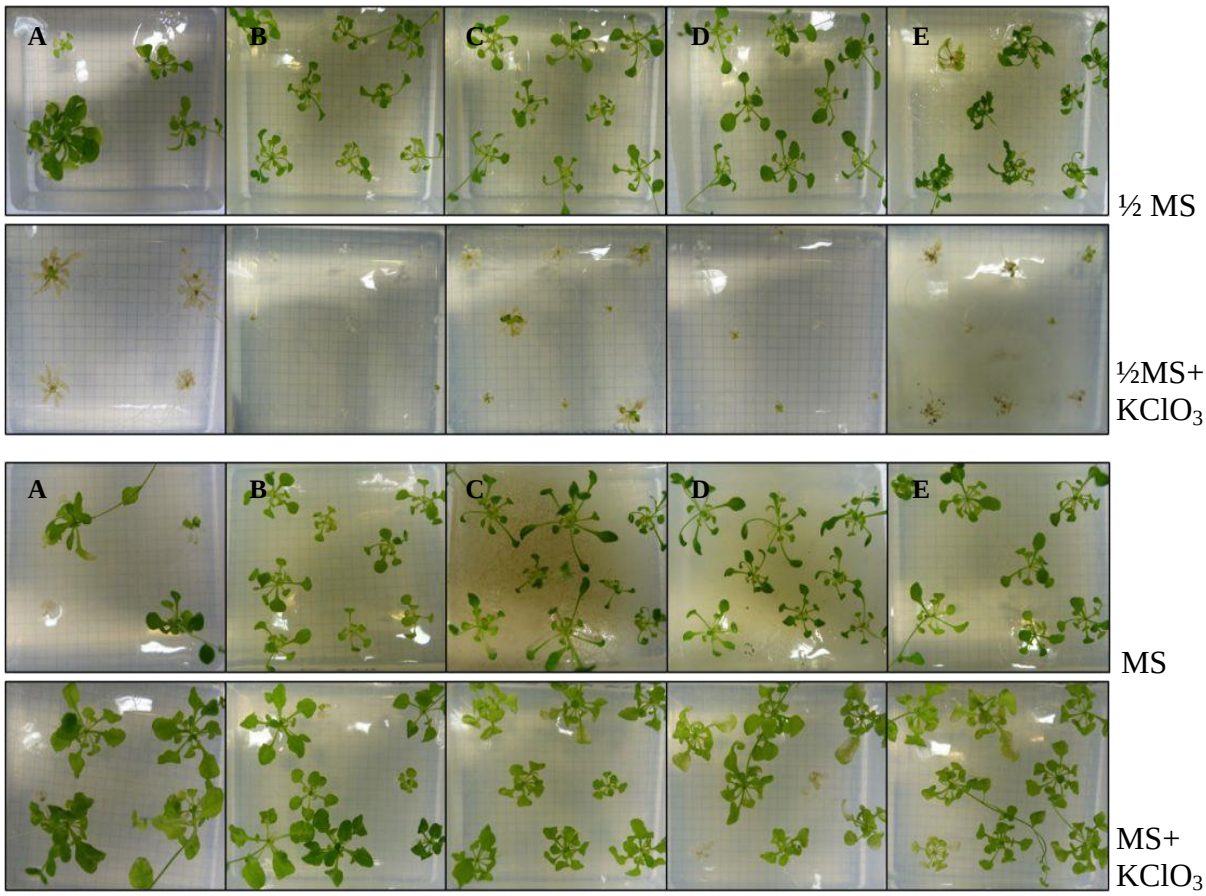


Figure 11. Plants after 30 days on different MS media with 5 mM $KClO_3$ or without 5 mM $KClO_3$. Plants of A) *atstr1* B) *atstr2* C) *atstr14* D) *atstr15* and E) wtcol were grown for four weeks on ½ MS or MS medium then transferred in fresh medium or in medium containing 5 mM $KClO_3$ and were observed for 30 d.

To foreclose that the transfer of the plants was responsible for dead plants wtcol plants were sown on MS and ½ MS medium twice in boxes. The plants of one box transferred after four weeks on fresh medium while the other stayed. After 30 days all plants survived. There was no difference between the transferred and the remaining plants (data not shown).

DISCUSSION

In *Arabidopsis thaliana* 20 putative members of Str were identified. In this work T-DNA insertion mutants of AtStr1, AtStr2, AtStr14 and AtStr15 have been used to investigate the biological function of Str proteins. With PCR the plants get screened if the mutation was homozygous. The mutants of AtStr2, AtStr14 and AtStr15 were screened in former studies (Bartels, 2006) so it was just necessary to screen AtStr1 more often to get homozygous seeds (Table 3). After finding homozygous seeds they were sow on MS or ½ MS medium with different supplemental substances to see the mutants behave during germination. The germination rate of wtcol was in the most experiments near 50% normally it has to be near 100%. It seems that the vitality of the seeds was not good anymore. The experiments have to be repeated with new seeds with a good vitality. Conformable with the results of Mao et al. (2011) which also work with *atstr1* (SAIL_69_D10), in our experiments the germination rate was about between 20-40% (Fig. 3). In the experiments of Mao coworker (2011) the germination rate was about 25%. On MS medium containing 1 mM TS the germination rate increased till 90% (Fig. 3). Sulfur is an essential element for both prokaryotic and eukaryotic cells. Plant cells take up sulfur from the external environment mostly as sulfate ion. Bacteria take it up as sulfate or thiosulfate compounds, and thiosulfate assimilation in bacteria has been extensively studied (reviewed in Ghosh and Dam, 2009). Although fragmented information can be found on thiosulfate in plants, reviews and research articles on the biochemistry and molecular biology of plant sulfur metabolism scarcely refer to it, reflecting a lack of knowledge on its importance to cell metabolism and to regulation of plant growth (Hell et al., 2010; Yi et al., 2010; Mugford et al., 2011). The highly increased germination rates on MS containing 1 mM TS of *atstr1* and also of *atstr2* and *atstr14* gives a hint that TS play an important role in plant development. In addition it gives an evidence that especially AtStr1 play a role in TS production when sulfite and 3-MP was present (Brychkova et al., 2013). In mammalian and invertebrates Str play a role in thiosulfate production from hydrogen sulfide (Hildebrandt and Grieshaber, 2008). A sulfur dioxygenase in the mitochondrial matrix oxidized persulfides to sulfite and a Str catalyze the transfer of a second persulfide from sulfide: quinon oxireductase to sulfite to produce thiosulfate (Hildebrandt and Grieshaber, 2008). Recent studies showed that ethylmalonic encephalopathy protein1 (ETHE1) encodes a sulfur dioxygenase in *Arabidopsis thaliana*. This protein plays a role in embryo and endosperm development (Holdorf et al., 2012). This hardened the evidences that Str play a role in thiosulfate production. With additional experiments this result has to be confirmed.

The experiment with 1 mM sulfite showed that *atstr2* and *atstr15* have the same germination rates like the wtcol nearly 100% but 74% of *atstr15* and 66% of wtcol plants were dead after six weeks. Of *atstr2* there were just 21% dead (Fig. 3). Sulfite, a less oxidized form of sulfate is an intermediate in the assimilation of sulfate and a potentially cytotoxic molecule, that if not rapidly metabolized can wreak havoc at the cellular and whole plant levels. It was shown that MST1 and MST2 catalyze the synthesis of the less toxic compound thiosulfate in the presence of 3-MP and sulfite (Brychkova et al., 2013). Although the gene of AtStr2 was knocked-out the survival rate of these plants was higher. This result suggests that another Str, e.g. AtStr1, resume the function of the AtStr2. Vice versa this did not work because the germination rate of *atstr1* was as low as on control medium (20%). The germination rate of *atstr1*5 on MS containing 1 mM sulfite was near 100% like on MS medium but 74% of the plants died after six weeks (Fig. 3). It seems that sulfite did not metabolized and had a toxic effect on the plant, although AtStr1 and AtStr2 could catalyze the synthesis of the less toxic compound thiosulfate. It looks that AtStr15 had also a function in the sulfite network. Another reason could be that 3-MP is missing but when this is the case it is strange that the plants of *atstr2* survive. Future investigations have to take a closer look on the function of Str at the sulfite network.

The experiments on MS medium with low and high concentration of sulfate as well as with low and high concentration of phosphate showed low germinations rates with a high standard deviation (Fig. 3). There were some problems with water in the plates. May be this water perturbed the development of the plants. To verify the results, more repetitions have to be done. Especially the experiment with TS has to be repeated because this was done once due to a defective climate chamber.

The Northern blot analysis of *atstr2* and wtcol plants exposed to different stress conditions labeled with different probes just show an effect between mutant and wtcol on the membrane labeled with AtStr2 probe. This result indicates that the AtStr2 gene is knocked-out completely. All other probes do not show any effects between mutant and wtcol plants. There were differences in expression between the stress conditions except the expression of Trxo1. The band of the control from the AtStr2 mutant plant is not detectable in comparison to the wtcol plant there is a clear band detectable. For the samples treated with 0.15 M NaCl there is a considerably expression at mutant and wtcol wherein the band of the wtcol is stronger (Fig. 5E). Also the expression of AtStr1 was increased at this condition but with no

differences between mutant and wtcol (Fig. 5A). This could indicate that these proteins interact because of the increased expression of both. Henne et al. (Chapter 3) showed with bimolecular fluorescence complementation (BiFC) that AtStr1 and Trxo1 interacts. We also assume that one Str can stand for another Str especially AtStr1 for AtStr2 and vice versa because they have a high degree of homology (68,6% identity and 76% similarity (Papenbrock et al., 2010)). But there was no indication at the expression pattern of AtStr1. No increased expression of AtStr1 was observable in the AtStr2 knock-out plants. It rather appears that AtStr14 had a slightly higher expression in the AtStr2 mutant plants (Fig. 4C). This is particular obvious in the control, 24 h at 4°C and 0.15 M NaCl. For a final conclusion the experiments have to be repeated. Furthermore a hybridization with probe AtStr18 is needed because AtStr2 and AtStr18 are localized in the cytosol while AtStr14 was plastidic and AtStr1 was a mitochondrial Str (Bauer et al., 2004). It seems more consequential that a Str of the same compartment take over the function of another Str. If there were differences in the expression pattern of Trxh1 it was not clearly visible. Trxh1 is the interaction partner of AtStr2 (Chapter 3) so it will be interesting to see if the expression gave a hint for this interaction.

The Northern blot analysis of AtStr14 also shows a complete knock-out of the AtStr14 gene (Fig. 4B). It seems that the metabolism is not intact anymore at treatment with 1 M NaCl because there is no expression of AtStr2, AtStr14 and Trxf1 (Fig. 6, sample 3 and 4). This result was compatible with the optical observation of the plants which really looked unhealthy. The membrane hybridized with probe AtStr2 showed a strong band at the sample treated 24 h at 37°C. This indicates that AtStr2 play a role in response to heat stress. Giuliani et al. (2007) found Str in the hyperthermophilic bacterium *Aquifex aeolicus* which prefers temperature of 85°C. It seems possible that a Str is active under heat stress. To prove this hypothesis more experiments with heat stress need to be done in the future. Looking on the membrane hybridized with probe Trxf1 (Fig. 5C) it is recognizable that the two control groups (four days and seven days) show different expression patterns. AtStr14 mutant and wtcol showed the same expression at four day control group but at seven day control group wtcol showed a weak expression while the mutant show a strong expression like the four day control (Fig. 6C). It seems that this short time period is enough to change the expression of Trxf1. An expression analysis during aging would clear this assumption. Trxf1 showed no expression at the seven day darkness condition. In combination to former results (Henne unpublished) it seems that Trxf1 was not active during darkness.

The stress conditions for the experiments with *atstr2* and *atstr14* were chosen on the background of the microarray studies on Genevestigator (www.genevestiagtor.com, 27th July 2010). For the salt experiments concentration of 150 mM were chosen. To get a strong stress condition the plants were treated with NaCl solution till the concentration of 1 M NaCl. This high salt concentration was too high and the plants were dead after 4 d. This high concentration is also for halophytes too high.

The measurement of the Str activity was done with the total protein extract and with 3-MP as sulfur donor and cyanide as acceptor. Because the activity was measured with the total protein extract it was not possible to differentiate the differences in the activity because *Arabidopsis thaliana* had 20 putative Str.

The Str activity in *atstr1* on MS and ½ MS medium was about 80 nmol min^{-1} mg^{-1} which was significant lower than the activity in wtcol (150 $nmol^{-1}$ mg^{-1}). Mao and coworker (2011) measured a Str activity of 118 nmol min^{-1} mg^{-1} for wild-type and 18 nmol min^{-1} mg^{-1} for *atstr1* which was much more smaller than our measurements but they work with a different mutant (SALK_155793). On ½ MS medium *atstr15* had also a decreased Str activity (80 nmol min^{-1} mg^{-1}) which was on MS not the case. It seems that the higher nutritional supply in MS medium increase the Str activity.

Yang et al. (2003) have reported that *Ntdin,* a possible tobacco orthologue of SEN1 (AtStr15) in *Arabidopsis thaliana* (56.8% identical) is involved in molybdenum cofactor (MoCo) biosynthesis in tobacco. Transgenic tobacco plants with suppressed Ntdin were found to have low nitrate reductase activity, an enzyme that requires MoCo, and enhanced tolerance to chlorate, a substrate analog of nitrate reductase. They have also suggested that AtSen1 have the similar function and may be involved in MoCo biosynthesis. The chlorate sensitivity test conducted according to Yang et al. (2003) was done with the mutants and wtcol. After 30 days on ½ MS or MS medium with and without chlorate no differences between mutants and wtcol were observable. If AtStr15 or one of the other tested Str played a role in MoCo biosynthesis the mutants had to grow better than the wtcol on chlorate medium. This was not the case and confirms the result from Schenk et al. (2005). It was conspicuous that the plants on MS with chlorate survived while the plants grown on ½ MS with chlorate died. Apparently the plants could handle the chlorate stress better with nutritional supply of MS.

The reduced glutathione is synthesized from glutamate, cysteine and glycine in two ATP-dependent reactions catalyzed by γ-glutamyl cysteine synthetase (γ-ECS) and glutathione synthetase (GSase) (Foyer et al., 2001). 162 nmol g^{-1} FW was measured in the plants grown on MS medium in all plants (mutants and wtcol). Typical values for leaf glutathione of the order of 200-600 nmol g^{-1} FW with the exact value depending on developmental stage and nutritional/light regime (Noctor et al., 2011). These results showed that the mutation did not have any effect on the GSH production because on MS medium wtcol and mutants have the same GSH content. Only differences on the different media were observable. On –S medium the mutants showed a low GSH content. GSH is the major reservoir of non-protein reduced sulfur in plants (Foyer et al., 2001). The mutants cannot work with this small amount of S while wtcol had the same GSH content like on MS medium. On MS medium supplemented with 3 mM sulfate the GSH content of *atstr1* and *atstr2* is lower than in wtcol or *atstr14* and *atstr15*. Whereas in *atstr2* was the lowest concentration. This indicates that AtStr2 played a role in sulfur incorporation into GSH. The biosynthesis of GSH depends on the availability of cysteine. It was expected that the GSH content on MS medium with cysteine is quite higher but the wtcol samples showed less than half GSH content than on MS medium. And also the concentrations in the mutants were lower than on the control medium (MS medium).

The chl a and chl b content and carotenoid content was determined spectrophotometrically. Looking on the calculated chl a contents, *atstr1* showed the smallest amount but looking on the ratio between chl a and b *atstr1* showed a ratio of 3.52 which was higher than the standard ratio of 3. The measurement was just done once so it is difficult to say if this is the reason of the mutation. To verify the results of the chlorophyll and carotenoid determination the measurements have to be repeated.

Additional the plant extract from the mutants and wtcol were separate in a Capillary Electrophoresis (CE) (MDQ Capillary Electrophoresis system, Beckman Coulter, Krefeld, Germany) with a MDQ-PDA detector. The separation of the samples took place at 14 kV and 22°C for 13 min. The wavelength for detection was 350 nm. Comparing the chromatograms of all samples no differences were detected. So it was not necessary to distinguish the single compounds in the plant extract.

This work and recent results from other working groups revealed more details and evidences that Str play a role in the sulfur metabolism especially in the sulfite network. In the future

more investigations have to be done with the main attention on these results to confirm this role of Str in the metabolism of higher plants.

CHAPTER 5

GENERAL DISCUSSION

Sulfurtransferase

The first rhodanese activity in plants was already found in 1938 (Gemeinhardt, 1938) however, closer investigations were undertaken much later. Sulfurtransferases (Str) have been characterized in many eukaryotic and prokaryotic species investigated to date. Although a lot of investigations were done on this enzyme family the physiological role is unknown till now. The redundancy supports the notion that Str are involved in distinct cellular processes. However, this makes it difficult to attribute a defined *in vivo* function just by analyzing the phenotype of the corresponding mutant. Characterizing the physiological role of Str in an organism is a challenge due to the abundance of potential Str/Str-like proteins within the same genome. Double knock-out mutants of Str could clarify this. Mao and coworkers (2011) worked with T-DNA mutants of AtStr1 and AtStr2 and also tried to work with a double mutant of these Str. Without both Str the plants were nonviable. They found out that AtStr1 and AtStr2 play a role in the embryo and seed development whereat AtStr1 played a more important role than AtStr2 (Mao et al., 2011). In mammalian and invertebrates Str plays a role in thiosulfate production from hydrogen sulfide. A sulfur dioxygenase in the mitochondrial matrix oxidized persulfides to sulfite and a Str catalyze the transfer of a second persulfide from sulfide: quinon oxireductase to sulfite to produce thiosulfate (Hildebrandt and Grieshaber, 2008). Recently, a sulfur dioxygenase in plants, ETHE1 (ethylmalonic encephalopathy protein1), was shown to be essential for embryo and endosperm development (Holdorf et al., 2012). ETHE1 is localized in mitochondria and exhibit sulfur dioxygenase activity (Holdorf et al., 2012). Therefore it is possible that AtStr1, which is also located in mitochondrion, catalyze the transfer of persulfide to sulfite to produce thiosulfate in plants. The germination rate of nearly 90% on medium with TS of *atstr1* proved this. When *atstr1* was knocked-out the germination rate was low (20-40%) the loss of thiosulfate production can be the reason. Is TS available in the medium the plant developed normally. Another working group showed that Str played a role in the sulfite network. In presence of sulfite and 3-MP, Str produced thiosulfate (Brychkova et al., 2013). Further investigations on this mechanism especially the TS production can prove this hypothesis.

As mentioned before it is difficult to identify specifically the *in vivo* function just by analyzing the phenotype of mutants and wtcol because of the function in many cellular processes and the abundance of Str in the whole genome. The four T-DNA insertion mutants (*atstr1, atstr2, atstr14* and *atstr15*) we analyzed did not show any differences in the phenotype in comparison to the wtcol plants. The development was slightly delayed in the mutant plants but there was no indication that Str play a role in developmental procedures of vegetative and reproductive organs. Quantitative analysis of the development revealed that wtcol produced more than twice as many siliques than the mutants (Chapter 4, Fig. 2B). The mutants of AtStr1 and AtStr2 showed the smallest amount of siliques (Chapter 4, Fig. 2B) which fits to the results mentioned above. This delayed development was also observable in the length of the stems. The stems of the mutants were significantly smaller than the wtcol stems. Only in the first weeks the section of the rosette was smaller after time *atstr1* and *atstr2* had the same size of the rosette as wtcol. Only *atstr14* and *atstr15* had a highly significant smaller size of the rosette. The Str activity is higher in older plants than in younger plants and leaves (Papenbrock and Schmidt, 2000a).

The expression studies done in this work aimed to find indications whether one Str can take over the function of another. The hybridization with Trx probes should give some indications whether Trx have parallel expression patterns as Str. Because of the high similarity of AtStr1 and AtStr2 on the protein level (78%) we assume that they can replace each other although they are localized in different compartments. The expression studies done with *atstr2* in Chapter 4 did not show any evidence that AtStr1 take over the function of AtStr2. AtStr1 did not show a stronger expression when AtStr2 was knocked out. Interestingly, a stronger expression in *atstr2* was observed in *atstr14*. Under 24 h drought and salt stress (150 mM) the expression of *AtStr14* was more than twice as strong in *atstr2* in comparison to wtcol (Fig. 5C). Considering the membrane of *atstr14* hybridized with an *AtStr2* probe it seems that the expression of *AtStr2* is also slightly higher than in the wtcol sample under 24 h at 37°C and 0.5 M NaCl but loading of the RNA was not completely equal with *atstr14* samples. To verify this result the Northern blots have to be repeated or a quantitative PCR (qPCR) has to be done. A different expression between mutant and wtcol samples was not observed in this study. Former studies on the expression of *AtStr15* showed that it was induced at various stress conditions (Bartels et al., 2007b). In our interaction study *AtStr15* showed an interaction with four different Trx in one compartment (Chapter 3) whereas all other Str showed an interaction with just one Trx. Caplan et al. (2008) showed that a homologue of AtStr15

NRIP1 interacts with the p50 replicase of TMV and the whole complex was transported to the nucleus and an immune response followed. These results showed that AtStr15 is a very interesting protein. But it was not possible to purify the recombinant protein. Many attempts like cloning in different vectors were not successful and an enzyme activity assay could not be done.

Ntdin a homologue to *AtStr15/AtSEN1* in *Nicotiana tabacum* is associated with senescence and play a role in Moco-biosynthesis (Yang et al., 2003). Based on these results a chlorate sensitivity test was done to investigate if AtStr15 and also AtStr1, AtStr2 and AtStr 14 play also a role in Moco biosynthesis. Between mutant plants and wtcol plants no differences were observable. These results confirm former analysis (Bartels, 2006). Just differences between the media were observed. All plants grown on MS medium, regardless of a chlorate addition or not, survived. Whereas the plants grown on ½ MS with chlorate died while the plants on ½ MS survived. The plants on MS medium were more tolerant to the chlorate stress probably because of the higher nutritional supply.

Interaction of sulfurtransferase and thioredoxin

In this work functional investigation of Str were done. The interaction between Str and Trx was detected in former investigations (Nandi et al., 2000; Ray et al., 2000; Nagahara et al., 2007; Sabelli et al., 2008). A deeper look in the interaction of Str and Trx in *Arabidopsis thaliana* showed a compartment-specific interaction. With BiFC analyses different Str from different compartments (Str1/mitochondria, Str2/cytosol, Str14/chloroplast, Str15/chloroplast, Str16/chloroplast and Str18/cytosol) were tested with different Trx from the same compartments (Trxh1/cytosol, Trxh3/cytosol, Trxy1/chloroplast, Trxm1/chloroplast, Trxm4/chloroplast, Trxf1/chloroplast, Trxo1/mitochondria and Lilium5/chloroplast). Also a compartment crossed analysis was done. The results showed that the interaction between Str and Trx is specific. Only one Str interacts with one Trx in the same compartment. AtStr15 was an exception; this protein interacts with four different Trx proteins but in the same compartment. A compartment crossed interaction was not observed maybe due to strict localization in one compartment making physical interaction impossible. Additionally the BiFC analysis was done with mutated Trx. Either one or both cysteines in redox site were changed by serine. The mutated Trx did not show any interaction with Str. The results of the

BiFC analysis were shown in Chapter 3. The presence of Str and Trx in the same cellular compartment can be considered as evidence in support of their interaction, and subsequent role in the maintenance of redox homeostasis.

Confirming the results of BiFC analysis two enzyme assay were done on the background of NADPH oxidation (Nandi and Westley, 1998; Nandi et al., 2000) and H_2S production (Siegel, 1965). The test of NADPH oxidation with Str2 and Trxh1 was auspicious in the first measurement but the result could not be reproduced once again. Nandi et al. (2000) used thiosulfonate as substrate for Str while in our assay 3-MP was used. This may be is a reason why this test did not work as expected. The H_2S test was done many times with many changes for optimization purposes. Finally the assay as described in Chapter 3 could be established and done with reproducible results. These results did not show any correlations between the BiFC results and the enzyme assays. There was no pattern recognizable. AtStr1 showed in each approach the highest H_2S production and also the highest Str activity with 3-MP while the activity of the other Str were lower (Fig. 3, Chapter 3). The enzyme assay was not conducted with the mutated Trx so far. The BiFC assays show the interaction *in vivo*. It seemed that there were more compounds necessary in the enzyme assay than we put inside. Another reason for these results could be the wrong substrate. Investigations on the *in vivo* substrate will be helpful to optimize the enzyme assays. Cross-linking experiments with AtStr16 and AtStr18 and Trxy1, Trxh1 and Trxh3 was started. The first results confirmed interaction results but more work have to be done. Also other tests could be done to confirm this BiFC interaction by chemical or physical methods.

CONCLUSION

It is clear that Str are involved in the regulation of metabolism by functioning in a range of processes such as protein biosynthesis, co-factor biosynthesis, regulation of redox homeostasis and protection against biotic and abiotic stress and many more. The complete function and importance of these ubiquitous enzymes is still unknown. Investigation on the possible substrates *in vivo* will give more indications of the physiological role of Str. Additional work on the interaction of Str with Trx and other proteins has to be done by different proteomic approaches. The research which was done in the recent years gives a small insight in the function of Str.

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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.

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PUBLICATIONS AND POSTER

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