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Sulfurtransferase and thioredoxin specifically interact as demonstrated by bimolecular fluorescence complementation analysis and biochemical tests

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ABSTRACT

Sulfurtransferases (Strs) and thioredoxins (Trxs) are members of large protein families. Trxs are disulfide reductases and play an important role in redox-related cellular processes. They interact with a broad range of proteins. Strs 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 protein–protein interactions of Strs with members of the Trx family. We are interested in investigating the specificity of the interaction between Str and Trx isoforms. In order to use the bimolecular fluorescence complementation (BiFC), several Str and Trx sequences from *Arabidopsis thaliana* were cloned into the pUC-SPYNE and pUC-SPYCE split-YFP vectors, respectively. Each couple of 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-active site of Trxs abolished the interaction signal. Therefore, the redox site is not only involved in the redox reaction but also responsible for the interaction with partner proteins. Biochemical assays support a specific interaction among Strs and certain Trxs. Based on the results obtained, the interaction of Strs and Trxs indicates a role of Strs in the maintenance of the cellular redox homeostasis.

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1. Introduction

Rhodanese activity, the transfer of a reduced sulfur from thiosulfate to cyanide, was found in many organisms. The main physiological function of these rhodanese/sulfurtransferase (Str) proteins was described to be cyanide detoxification [43]. Later bioinformatic analysis demonstrated that proteins containing a Str/rhodanese domain are present in all three domains of life [8]. 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 Strs in *Arabidopsis thaliana* consists of 20 proteins containing one or two rhodanese domains, and they are located in different cellular compartments [31]. 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 Strs in plants remains unresolved so far. In recent years, the results of several independent research approaches indicate that Strs interact specifically with different proteins [13,1], but mainly with thioredoxins (Trxs), regulatory proteins involved in cysteine-thiol disulfide exchange [27,26,37]. All together more than 40 Trxs and Trx-like proteins were identified in *Arabidopsis* containing a five amino acid redox site with two cysteine residues (WCXXC) and being also located in the different compartments of the cell. Unlike animal and yeast counterparts, the function of Trxs

Abbreviations: Str(s), sulfurtransferase(s); Trx(s), thioredoxin(s); YFP, yellow fluorescent protein

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in plant mitochondria is largely unknown [22]. There are two major questions: (1) why are there so many Trxs and Trx-like proteins in plants in comparison to other taxa, and (2) what is the biochemical basis for their target-protein specificity [19]. Therefore, more information about the redox interactome is needed.

First results indicating a Str/Trx interaction came from enzyme activity measurements in different species. Bacterial and mammalian Strs from mitochondria catalyze the direct oxidation of reduced Trx demonstrating that Trxs could be a physiological substrate for Strs [27,33]. In animals, at least one Str/rhodanese isoform could serve in the detoxification of intra-mitochondrial oxygen free radicals [28]. The redox state regulates 3-MP-dependent Str activity from rat at the enzymatic level and 3-MP Str was shown to maintain the cellular redox homeostasis [25]. This hypothesis was supported by a proteomic affinity approach demonstrating an interaction of Trx with 50 mitochondrial proteins, among them AtStr1 [2]. 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 more negative redox potential than glutathione [25,26].

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 Fig. 1. There exists a family of six NADP-Trx reductases in *Arabidopsis* [22]. In the plastids three out of four proteins are ferredoxin-dependent Trx reductases, in the cytoplasm and the mitochondria the proteins are NADP-dependent Trx reductases [18].

The goal of this study is to analyze whether there are interactions among Str and Trx proteins, to explore the specificity of the interactions, and to frame the molecular basis thereof. We concentrated on the analysis of a selection of Str that had been previously localized by GFP fusions [5]. With respect to the Trxs representative members have been chosen that are present in different compartments of the cell. Several fundamentally different methods were applied to clarify this postulated interaction. These methods are based on close physical proximity as a prerequisite in bimolecular fluorescence complementation (BiFC) as discussed in Bracha-Drori et al. [10] and Walter et al. [42]. In the biochemical tests the interaction could either be a protein–protein interaction or a weaker, transient substrate-channeling interaction. Results indicate a compartment- and partner-specific protein–protein interaction for some Str/Trx pairs and will help to elucidate the *in vivo* functions of Str proteins in plants.

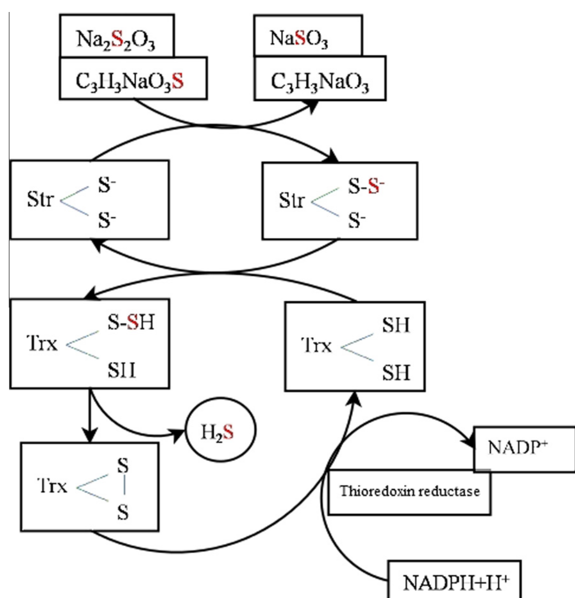

Fig. 1. Schematic reaction cycle of Str, Trx, and NADP-dependent Trx reductase (modified after [28]).

2. Results

2.1. Sulfurtransferase interacts with thioredoxin as demonstrated by bimolecular fluorescence studies

Different combinations of pUC-SPYNE and pUC-SPYCE vectors carrying mitochondrial (AtStr1, Trxo1), cytoplasmic (AtStr2, AtStr18, Trxh1, Trxh3), or plastidic (AtStr14, AtStr15, AtStr16, Trxy1, Trxm1, Trxm4, Trxf1, Lil5) Str and Trx and mutated variants thereof were transiently transformed into protoplasts isolated from *Arabidopsis* leaves (Table 1). Proteins that are postulated to interact were fused to unfolded complementary fragments of the yellow fluorescent reporter protein and expressed in living cells. Interaction of these proteins will bring the YFP fragments within proximity, allowing the reporter protein to reform in its native three-dimensional structure and emit its fluorescent signal [16]. 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 [42] and a clear signal in the nucleus was visible (Fig. S1). Control experiments using each protein for co-transformation with an empty vector did not reveal any fluorescence (Fig. S2; data not shown). All results of protein pairs tested by BiFC are summarized in Table 2.

All positive interaction pairs were localized in the same compartment as was previously shown or predicted for the single proteins [5,22]. 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). The analysis of AtStr16/Trxy1 (Fig. 2C) is possibly representing a physical interaction, but precise localization of the Str16/y1 complex will need additional investigations. 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 (TPTrxy1withTrxh1) but neither with the cytoplasmic AtStr2 nor with the plastidic AtStr15 a positive signal was obtained, although the cytoplasmic Trxh1 usually shows an interaction with AtStr2. Except Trxm4, all investigated Trx proteins 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% identity and 65% similarity. Their redox site is identical (WCGPC) but the features around the protein disulfides differ.

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 assumed 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 (Table 3) 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, Trx-

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